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Bacteriophage: Bioengineered Bacterial Detection and Applications

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Bacteriophage: Bioengineered Bacterial Detection and Applications

A Dissertation Presented

by

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DEDICATION

This work is dedicated to my girls: my daughters, Severine and Lys, and my wife Jennifer.

In Amherst, we grew as a family.

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I would like to thank Dr. Nugen for inviting me to his lab to embark on what has been a life changing adventure. Your counsel has helped me develop as a researcher, motorcycle rider, parent, and friend. For that, and a few dinners I never paid you back for, I am forever indebted. Thank you Sam.

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ABSTRACT

BACTERIOPHAGE: BIOENGINEER BACTERIAL DETECTION AND APPLICATIONS

FEBRUARY 2016

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Bacteria are ubiquitous and vital constituents of our environment, our foods, and our bodies. A small percentage of this vast, microbial population is pathogenic to humans, but represents a significant burden on public health. There is a current public health focus on two subgroups: foodborne pathogenic bacteria and antibiotic resistance bacteria. A key challenge for public health is the rapid identification of these bacteria to prevent their consumption and to ensure proper treatment for infections. This challenge calls for the development of novel, low-cost diagnostics that combine sensitivity and accuracy with speed and ease-of-use.

Bacteriophages represent rapid, readily targeted, and easily produced probes for the detection of bacterial pathogens. Furthermore, modern molecular tools have allowed researchers to make significant advances in the bioengineering of bacteriophage to further improve speed and sensitivity of detection. Our work here demonstrates the successful bioengineering of bacteriophage to create platforms that: (i) enable multiplex detection of bacterial pathogens; (ii) allow for the rapid detection of antibiotic resistant bacteria; (iii) and can be used synergistically with other diagnostic platforms, like paper-fluidics, plate readers, and MALDI-TOF.

In particular we demonstrate: (i) the modification of T7 bacteriophage to carry TEV protease enabling proof-of-principle detection of *E. coli* in 3 hours after a primary enrichment via TEV protease activity using a fluorescent peptide and using a designed target peptide for MALDI-TOF MS analysis; (ii) the development and use of a phage amplification-based lateral flow assay for the detection of low levels of *E. coli* in less than 7 hours in both broth and water; and (iii) the modification of T7 phage to carry *phoA*; enabling the rapid detect of low levels of bacteria and their antibiotic susceptibility in under 6 hours. These phage-based platforms could be readily adopted in many labs without significant capital investments and can be translated to other phage-bacteria pairs for further detection. Further research into the bioengineering of phage-based reporters and demonstrating their incorporation into common and novel diagnostics platforms, will highlight their potential and ideally result in the creation of diagnostics that impact public health.

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CHAPTER 1
BACTERIOPHAGE: POTENTIAL APPLCATIONS FOR RESOURCE-LIMITED DIAGNOSTICS

1.1 Introduction

Bacteria are an integral part of our world. They are present in the environment that surrounds us, the food and water we consume, and are vital inhabitants of our bodies. Of this vast bacterial population, a small percentage is pathogenic to humans, but this group represents a significant burden on public health. At both the national and global level, there has been increasing focus by public health agencies on the prevalence of foodborne pathogenic bacteria[1-3], as well as the rise and dissemination of antibiotic resistance bacteria[4-7]. A key challenge in both these areas is the rapid identification of these bacteria to: (i) prevent the distribution and consumption of contaminated foods and water[8-10]; or (ii) to ensure rapid and proper treatment post-infection[11]. This challenge is further acerbated by global and within-country disparities in economic, technological, and regulatory capabilities[12-14]. For example, implementation of a mandatory PCR-based bacterial screening of foods by producers could be feasible in resource-rich, politically stable countries, but impractical for small producers or within countries that lack the basic resources required like electricity, access to supplies, financials, and as educated workforce. These added challenges in resource-limited settings, have driven research into the development of "lab-on-chip" technologies[15] aimed at addressing issues with manufacturing, detection, automation, and power source to create diagnostics that are simple, cheap, and reliable and thus amenable to adoption in such settings.

Paper-fluidic [16, 17] and microfluidic [18, 19] based devices have proven themselves to be effective platforms for the detection of chemicals and pathogenic organisms of interest in resource-limited settings [14], and there is a large body of research applying them for detection of various bacterial, chemical, viral agents[20-23]. The home pregnancy is perhaps the best recognized embodiment of a paper-

fluidic device, and highlights the simplicity, cost-effectiveness, and reliability of the lateral flow assay (LFA). The recent introduction of the home use HIV test kits[24], further demonstrates the successful adoption of the lateral flow platform in real world diagnostics addressing public health. These technologies have begun to replace more traditional culture based methods, in both primary and third party testing laboratories, to enable rapid screening of samples. Commercially available examples of these LFA based products are the Lateral Flow System by DuPont (Wilmington, DE) and Neogen's Reveal 2.0 product line, both of which have dipstick assays for the detection *E. coli* O157:H7, *Salmonella* spp., and *Listeria* (Neogen, Ann Harbor, MI).

1.2 The Lateral Flow Assay

The schematic of a lateral flow assay is fairly simple. In its most common form, the LFA consists of a nitrocellulose membrane, containing a test line, control line, with a sample and conjugate pad at one end, and an adsorbent pad at the other. The test line is composed of a capture agent against the target molecule being measured. Typically, the capture agent is either: (i) an antibody against the specific target, like a protein, bacterial surface antigen, or chemical [20, 21, 25-27] ; or (ii) the capture agent can be a DNA aptamer against a specific target DNA sequence[17, 28-30]; though any capture system, like biotin-streptavidin, can be leveraged if appropriate. The sample pad, as its name implies, is where an aliquot of the test sample is placed. From the sample pad, the solution flows through the conjugate pad. If the LFA is a sandwich assay, the conjugate pad contains a secondary detector antibody or DNA aptamer against the test's target molecule. If it is a competitive assay, the conjugate pad contains the target molecule. In either of these assay formats, the conjugate pad contained detector or target molecule is linked to a reporter molecule. There are a various reporters molecules utilized, like colloidal gold or fluorescent particles[17, 21, 28], as well as enzymatic reporters, like alkaline phosphatase, that enable colorimetric, fluorescent, chemiluminescent, or electrochemical detection[21, 31-34]. In the sandwich assay,

the reporter-tagged detector binds the target molecule, which flows up the strip and is captured at the test line, producing a signal there. In the competitive assay, the untagged target molecule in the sample and the tagged target in the conjugate pad both run up the strip and compete for space at the test line. If the sample contains the target, there is an elimination or reduction in signal at the test line vs the control. The absorbent pad wicks up the solution, drawing the entire sample up past the test line.

1.3 Challenges for Lateral Flow Assays

While immuno-based LFAs have been successful applied for the detection many pathogenic organisms, there are potential drawbacks with the use of antibodies. The first is the expense and time to produce the antibody for a given target[21, 35]. Second is the limited specificity of antibodies there by limiting detection of a broad range of closely related molecules. It would be difficulty to develop a mixture of antibodies with a broad enough range to capture an entire species of bacteria, such as the generic *E. coli* which is commonly used as indicator of water quality[36] or *Salmonella* spp. which has over 2000 serotypes[37]. Thirdly, there are potential false positives that may result with antibody cross-reactivity, or in the case of bacterial antibodies attaching to components of non-viable cells, resulting in a false-positive test result[38, 39]. DNA-based LFAs also have the potential for false positives due to the presence of extraneous DNA from non-viable cells[40].

Another challenge for LFAs is the limited sample size and sample composition. Test sample sizes typically range from 50 µL – 100 µL, meaning that the target molecule may need to be at concentration levels not typically found in real world samples to be detected. LFA's by their design require a sample matrix that can flow up the test strip, which limits testing to sample matrices with low viscosity. Furthermore, sample color, clarity, ionic strength, and the presence of particulates, may also adversely affect flow and the ability to detect visual, fluorescent, or electrochemical signals at the test line. The sample may also contain compounds that inhibit PCR or other enzymatic reporters, leading to false

negatives. Conversely, the sample may contain endemic enzymes, fluorescent compounds, or genetic material that result in the production of false positives for a given method. LFA's are thus often with a separation methods to reduce the impact of this potential sample interference. Some of these methods have the added benefit of concentrating the target molecule [41]. LFAs have been combined with membrane filter, phase separation[42], and immuno-magnetic separation[43] steps to concentrate the target of interest. In some cases, an amplification step is also used to increase the target concentration or signal. Examples of amplification for LFA would be the use of a traditional bacterial enrichment step prior to testing, like in Neogen's Reveal 2.0 tests, or the use of a preliminary PCR step to amplify a target DNA sequence within a sample[21, 29].

1.4 Bacteriophage - Potential Applications

Bacteriophages (phages) are a class of virus that infects prokaryotic bacteria. They were first discovered in 1915, and initially used as antimicrobial agents but were quickly supplanted by antibiotics[44]. Phages play an important role in various industries like: dairy fermentation where they represent a significant product quality threat[45]; for bacterial strain typing based on phage susceptibility patterns[46-49], and as biocontrols for foodborne pathogens[50-53]. Our increased understanding of phage diversity, and the creative applications of modern bioengineering tools, has lead to renewed interest in bacteria phage. There are clearly opportunities to leverage these advances and apply phage to enhance diagnostics, like paper-fluidic LFAs, geared to resource-limited settings.

1.4.1 Bacteriophage as Detection Agents

Phages are becoming increasingly popular as probes in bacterial detection schemes[50, 54-56]. Phages have several potential advantages over other detection probes, like antibodies and DNA. First, phages are relatively easy to produce as they are readily propagated in the presence of their host[54] and

can be subsequently purified for use[57, 58]. A second advantage is that phage are host specific, and a single or multiple phage types can be used to target a specific bacterial strain, species, or group of closely related bacterial species[54, 56]. The successful use of reporter-tagged phage for bacterial detection has been demonstrated using both fluorescently[59-61] and enzymatically[62] tags. The VIDAS® UP by Biomérieux (St. Louis, MO) is a commercially available detection platform that utilizes reporter-tagged phage components as detector probes in an ELISA format for the detection of *Salmonella*, *E. coli* O157:H7, and *Listeria* with reported superior performance to PCR-based methods[63]. Phage-display schemes have also allowed for the development of novel phage-based probes by enabling the bioengineering of phage capsids to incorporate proteins with affinities to chemicals and molecule unrelated to bacterial detection[64].

1.4.2 Bacteriophage as Agents for Signal Amplification

The use of phages as a detection probes also allows for signal amplification, commonly referred to as phage amplification. In concept, a single phage adsorbs to and infects a single bacterial cell. The phage DNA is injected into the host, and through a series of mechanisms, it commandeers the metabolic machinery of the cell into the production of new phage. At the end of the infection cycle holin and lysozyme are produced, rupturing the cell wall, and releasing tens to hundreds of new phage into the environment. The *E. coli* phage T7, for example, has an infection-to-lysis time of 25 minutes under ideal conditions[65], and over 100 new phage released at the end of a cycle[66]. For comparison, *E. coli* cells take approximately the same amount of time to replicate under optimal growth conditions[67], so a traditional bacterial enrichment for amplification results in only 2-fold increase using versus the potential 100-fold increase in T7 phage in the same amount of time. There other phages with burst sizes greater than a 1000-fold[68], making their incorporation into bacterial detection schemes quite attractive. Furthermore, since phage replication requires healthy, growing bacterial cells, detection of phage amplification can be

used to specifically identify the presence of only viable cells in a sample. This is another advantage phage-based detection schemes have over immunological and PCR-based schemes[40, 69], which may detect extraneous antigens or DNA from non-viable cells resulting in higher false positive rates[9, 39, 40, 69].

Advances in molecular techniques, have enabled the combination of both phage and enzyme based amplification for bacterial detection. Phages have been genetically modified to express reporter enzymes with high turnover rates, enabling more rapid and sensitive detection. Luciferase has been successfully incorporated into several phage strains targeting bacterial pathogens like *E. coli* O157:H7[70], Yersinia pestis[71], and *Bacillus anthracis*[72]. Commercially, Sample6 (Boston, MA) has developed a phage-based bioluminescent enzymatic reporter platform for the detection of *Listeria* in environmental samples. In the literature luciferase appears to be a popular choice as an enzymatic reporter, but recent research has also shown the successful incorporation of enzymes like peroxidases[73], proteases[74], and alkaline phosphatase[75] as phage-mediated reporters.

1.4.3 Bacteriophage as Agents for Bacterial Separation and Concentration

Phage-host cell binding dynamics make phage an attractive alternative capture agent in place of antibodies in IMS based schemes[41]. Once a phage attaches to a cell, the bond is very strong and irreversible[76] in comparison to antibody-antigen bonds which are reversible[77]. Recent research has demonstrated the successful application of both phage[78] and phage components[79] as antibody replacements in these IMS based scheme. If the phage virions conjugated to magnetic beads are infectious, and the target cells captured viable, there exists the potential to create novel detection schemes that combine bacterial separation, concentration, and phage based amplification.

1.4.4 Bacteriophage as Agents for the Detection of Antibiotic Resistance

In the area of antibiotic susceptibility testing, the application of phage for the rapid detection of antimicrobial resistance is still in its infancy[11, 80, 81]. The FDA had approved a phage amplification-based lateral flow device for the rapid detection of methicillin resistant (MRSA) *S. aureus* [82], called KeyPath™ by MicroPhage Inc. (Longmont, CO) but the assay is no longer available[83]. DNA-based methods are quickly replacing traditional culture-based methods, and many of these new methods are automated, which is an adoption hurdle for the LFA format, like that used by MicroPhage, in high-throughput clinical testing labs that focus on antibiotic susceptibility testing. DNA-based antibiotic resistance systems have achieved expansive screening capabilities[84], but there remains a large reservoir of unknown antibiotic resistance genes[85] and resistance mechanisms[86, 87], making exhaustive DNA-based screening unattainable. There is also the issue of gene expression in resistance, as there is the potential for a gene conferring resistance to a given antibiotic to be present, but not properly expressed. DNA-based methods that screen for gene presence but not regulation could result in unnecessarily intensive treatments, with potentially more undesirable side effects, being employed to fight an infection. Phage amplification-based screening, because it relies on the phenotypic rather than genotypic state of the cells, sidesteps some of these DNA-based issues. Phage amplification requires viable bacterial cells, and thus amplification is contingent upon bacterial growth in the presence of the antibiotic. Nothing must be known about potential resistance mechanisms a priori to this phage-based testing. Phage amplification-based testing also allows for the implementation of several internal quality controls. The first is the elimination of false positives due to the cross-contamination of a test by an internal laboratory strain with resistance. If the contaminating strain is of a different species to that of the test strain, phage amplification will not occur whereas a traditional test looking for sample turbidity or a DNA–based test screening only for genes would result in a false positive signal in this scenario. There is

the issue of bacterial strains acquiring phage resistance, potential resulting in the very undesirable false negative for resistance. An assay control that includes growing the isolate in the presence of the phage but not the antibiotic would allow phage resistance to be flagged, and prompt for confirmation via alternative method. These advantages suggest there are still untapped applications for phage-based antibiotic susceptibility testing.

1.5 Conclusion

Phages represent multifaceted building blocks that can be incorporated as substitutes for, or in unison with other detection methods, to create powerful new diagnostics for the detection of bacteria. The ease of phage manipulation, production, and detection speed clearly highlights that there yet remains unrealized opportunities to leverage these phage-based components in diagnostics amenable to resource-limited settings. The passage of regulations like the Food Safety Modernization act, and the ever increasing extent of global trade and travel, will create further demand for these types of diagnostics. While phage-based diagnostics have begun to entering the market place, further research is needed to ensure the potential benefits of phage-based technologies for public health are fully realized.

CHAPTER 2
RESULTS OF A PILOT ANTIBIOTIC RESISTANCE SURVEY OF ALBANIAN POULTRY FARMS

2.1 Abstract

Goal: The global dissemination of antibiotic-resistant bacteria in food animals is a major public health concern. While many countries have implemented prudent antibiotic use policies and surveillance systems in both clinical and veterinary settings, there are no such systems in place in Albania and little is known about the levels of antibiotic resistance bacteria in food animals within the country.

Methods: A total 172 poultry samples were taken from six Albanian farms over a 3 month period and tested for the presence of *Enterobacteriacae.* A total of 91 bacterial isolates were obtained and were characterized by species (*Escherichia coli*, *Salmonella*, or other *Enterobacteriacae*) and by susceptibility to 11 antibiotics.

Results: *E. coli* and *Salmonella* isolates exhibited resistance to amoxicillin (86%, 64%), chloramphenicol (77%, 82%), ciprofloxacin (93%, 73%), cefotaxime (14%, 0%), gentamicin (12%, 0%), kanamycin (30%, 18%), nalidixic acid (91%, 73%), streptomycin (70%, 55%), sulphonamides (91%, 73%), tetracycline (95%, 73%), and trimethoprim (79%, 64%) respectively. Multidrug resistance to at least 4 antibiotics was observed in 95% of *E. coli* isolates and 82% of *Salmonella*.

Conclusions: Our data indicate that: (i) *Salmonella* and *E. coli* isolates from Albanian poultry farms exhibit high to extremely high levels of antibiotic resistance; (ii) *Salmonella* and *E. coli* isolates exhibit resistance to multiple antibiotics; and (iii) multidrug resistant profiles among *Enterobacteriacae* are geographically widespread. Implementation of prudent antibiotic use policies in food animals and related surveillance will be necessary to reduce the emergence, spread, and establishment of highly resistant strains across poultry farms in Albania.

2.2 Introduction

In response to rising rates of antibiotic resistance among clinical bacterial isolates and data linking antibiotic use to resistance development [12, 88, 89], leading public health organizations such as the World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC) in the United States of America, and the Royal Society of Medicine have published recommendations calling for: (i) prudent use of antimicrobials, (ii) improved surveillance of antimicrobial use and resistance, and (iii) awareness campaigns for both health professionals and the public on the control and risks of antimicrobial resistance [90]. Research has also highlighted the public health impact of veterinary antibiotic use and the movement of antibiotic resistant bacteria between animal and human populations [91, 92]. While antibiotics are commonly used to treat, prevent, and control diseases in food animals, as well promote growth, sufficient data detailing the amount and frequency of antibiotic use in animal husbandry is lacking [93]. Institutions such as the European Food Safety Agency (EFSA), European Centre for Disease Prevention and Control (ECDC), the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), and the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) in the United States, have been vital in illuminating the interplay between human and animal populations, antimicrobial use, and antimicrobial resistance. The monitoring systems supported by these institutions, however, are primarily focused on Western Europe and North America, obscuring the global picture of antibiotic resistance. Research suggests that the prevalence of antibiotic resistant bacteria is much higher in other regions [13]. The globalization of the food supply chain and increased global travel means that antibiotic practices and prevalence of antibiotic resistant bacteria within these countries will have public health implications well beyond their borders. It is therefore critical to develop a more precise picture of global antibiotic resistance, identify public health risks, and implement appropriate strategies for control where appropriate.

Albania is located in southeastern Europe, and in 2014 it became an official candidate for accession to the European Union. Albania currently lacks surveillance programs for antibiotic consumption and resistance, and so it is difficult to gauge the potential health impacts of increasing travel and trade. Recent surveys have found poor control and misuse of clinically important antibiotics [94, 95]. Agricultural exports are an important economic focus for Albania, but local food safety standards represent a hurdle for export growth [96]. There is little peer-reviewed published material on antibiotic use or antibiotic resistant bacterial levels in food animals in this country.

In this paper we report the results of a short-term pilot surveillance study initiated as part of a joint WHO-World Organization for Animal Health (OIE) project on "National Human and Animal Health Systems Assessment Tools and Bridges" to gain a preliminary understanding of bacterial resistance levels in food animals in Albania. This pilot survey looked at antibiotic resistance levels in *Enterobacteriacae,* with a focus on *Salmonella* and *E. coli*, isolated from across six poultry farms over a three month period. To put these results in context, we compared them to the antimicrobial resistance levels in poultry reported by EFSA for the EU. We hope the results of this pilot will call attention to the antibiotic related policies in Albania, and support the long-term implementation of robust surveillance and prudent use programs.

2.3 Materials and Methods

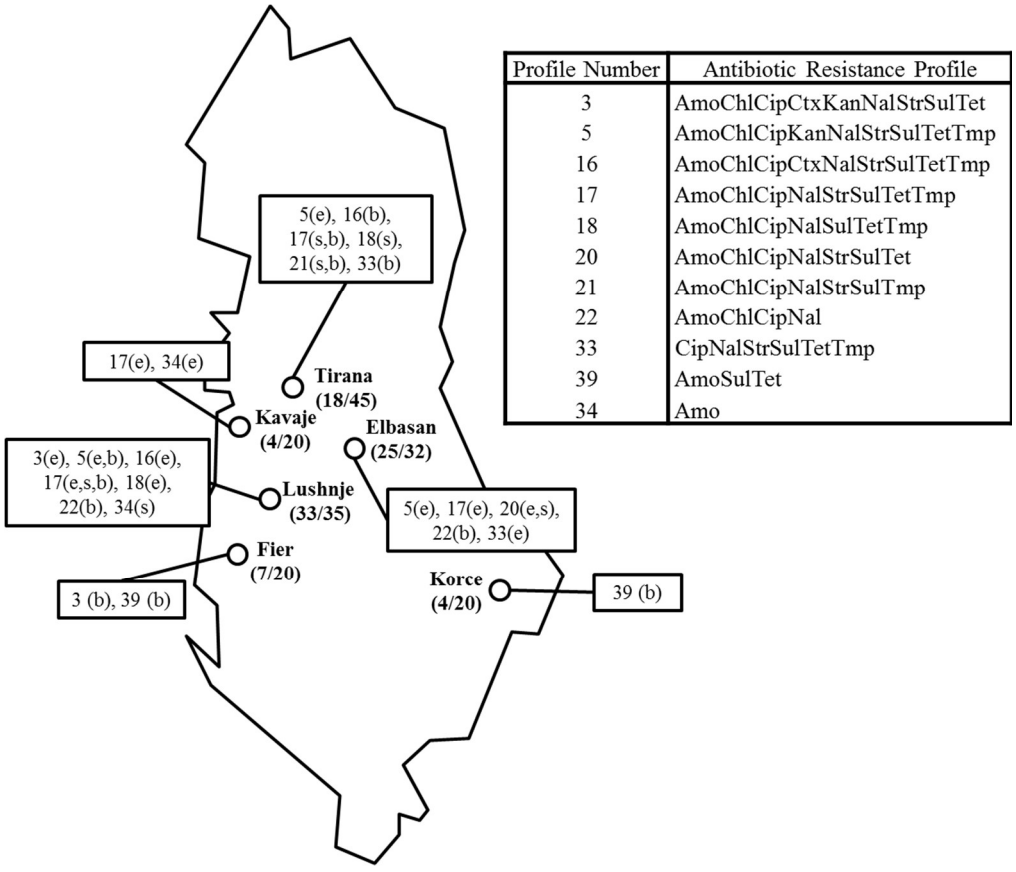
2.3.1 Bacterial Isolates

All isolates included in this study were obtained as part of a field survey examining the levels of antibiotic resistance on Albanian poultry farms. Isolates included in this study were cultured from 172 poultry samples of the neck skin, stomach, intestines & stomach, or cloacal swabs from slaughtered poultry. Samples were collected from six Albanian farms, during three visits to each farm from December 2013 to early February 2014 (Figure 2.1). Samples were processed using ISO 6579: 2002 "Microbiology of food and animal feeding stuffs – horizontal method for the detection of *Salmonella* spp." Subsequently, all colonies, including those that were not *Salmonella*-like on selective media, were characterized biochemically using API 20E (bioMérieux, Durham, NC, United States). For the purposes of this study, isolates were classified as *E. coli*, *Salmonella* spp., or "other *Enterobacteriaceae" (*e.g. *Klebsiella* spp., *Citrobacter* spp., etc). All *Salmonella* spp. were serotyped. A total of 91 bacterial isolates were cultured from the 172 samples, including 3 samples which yielded two isolates each. There were 84 negative samples.

2.3.2 Antibiotic Resistance Profiles.

All isolates were characterized for susceptibility to 11 antibiotics: amoxicillin (amo), chloramphenicol (chl), ciprofloxacin (cip), cefotaxime (ctx), gentamicin (gen), kanamycin (kan), nalidixic acid (nal), streptomycin (str), sulphonamides (sul), tetracycline (tet), and trimethoprim (tmp), using MIC Test strips (Liofilchem Diagnostics, Roseto degli Abruzzi, Italy). Interpretation was based on the epidemiological cut-off values (ECOFFs) from the European Committee on Antimicrobial Susceptibility Testing website [97]. *Salmonella* ECOFF's for kan and sul were listed as not determined, so the published *E. coli* ECOFF's values were used for analysis. The antibiotic resistance of isolates identified as other

Enterobacteriacae, were also interpreted using the reported *E. coli* ECOFFs. Once resistance to the 11 antibiotics was interpreted for all isolates, each unique resistance pattern was assigned a resistance profile number. This isolate data was used to determine the percentage of resistance to a given antibiotic, the frequency distribution of multidrug resistance, and the geographic distribution of species specific resistance profiles.



Profile Number	Antibiotic Resistance Profile
3	AmoChlCipCtxKanNalStrSulTet
5	AmoChlCipKanNalStrSulTetTmp
16	AmoChlCipCtxNalStrSulTetTmp
17	AmoChlCipNalStrSulTetTmp
18	AmoChlCipNalSulTetTmp
20	AmoChlCipNalStrSulTet
21	AmoChlCipNalStrSulTmp
22	AmoChlCipNal
33	CipNalStrSulTetTmp
39	AmoSulTet
34	Amo

Figure 0.1 Distribution of antibiotic resistance profiles across Albanian poultry farms. Farm location followed by (number of bacterial isolates / total number of samples taken). For each farm a box contains the profile number, and the letters in parenthesis indicate isolate type: e - *E. coli*, s - *Salmonella*, b – *Enterobacteriacae* with the profile.

2.4 Results and Discussion

This pilot antibiotic resistance surveillance survey encompassed six of the twelve large commercial Albanian poultry farms, over a three month period from December 2013 to early February 2014. These farms process an estimated 800,000 to 1,200,000 chickens a year. A total 172 poultry samples were taken, yielding 91 bacterial isolates. Isolates were characterized by species (*Salmonella*, *E. coli*, or other *Enterobacteriacae*) and by susceptibility to 11 antibiotics. All *Salmonella* isolates obtained were identified as *enteritidis* by serotyping. There were 92 neck skin samples taken, which yielded 1 *Salmonella*, 9 *E. coli*, and 22 *Enterobacteriaceae* isolates. Of the 20 stomach samples taken, 4 *Salmonella*, 7 *E. coli*, and 4 *Enterobacteriaceae* isolates were obtained. Of the 6 intestine samples taken, no *Salmonella*, 1 *E. coli*, and 3 *Enterobacteriaceae* isolates were obtained. Of the 12 stomach & intestine samples taken, 2 *Salmonella*, 2 *E. coli*, and 1 *Enterobacteriaceae* isolates were obtained. Of the 42 cloacal swabs taken, 4 *Salmonella*, 24 *E. coli*, and 7 *Enterobacteriaceae* isolates were obtained.

2.4.1 Bacterial Isolates from Albanian Poultry Show High to Extremely High Levels of Antibiotic Resistance

In 2012, the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) released the 3rd revision of critically important and highly important antibiotics for human medicine [98]. High levels of resistance to these antibiotics in food animal bacterial isolates have serious implications for general public health, safety of farm workers and their families, and the efficacy of veterinary treatments to control animal disease on-farm. Our data (Table 1) show there are reasons for concern about the antibiotic resistance situation in Albania.

Our pilot study found extremely high levels of resistance, > 70% as defined by the joint EFSA and ECDC EU summary report on antimicrobial resistance [7], to seven of the eleven antibiotics included (Table 2.1) in *E.coli* isolated from poultry. In contrast, the results of the 2012 EU summary report, no country reported *E. coli* from poultry exhibiting extremely high levels of resistance to any of the antibiotics tested [7]. Our study also found moderate levels of resistance to gentamicin and very high levels of resistance to streptomycin (Table 2.1), in contrast to low levels of resistance to gentamicin and high levels of resistance to streptomycin in the EU report[7]. Our study found moderate levels of resistance to cefotaxime, which was similar to the level of resistance in *E. coli* isolates from poultry reported in the EU report. *Salmonella* isolates from Albania poultry showed extremely high levels of resistance to 5 of the antibiotics included (Table 2.1). In contrast, no EU country reported *Salmonella* spp. isolates from poultry exhibiting extremely high levels of resistance to any of the antibiotics tested [7]. The EU summary did report low levels of resistance to cefotaxime and gentamicin [7], in contrast our study found no *Salmonella* isolates exhibiting resistance to either of these antibiotics.

Table 0.1 Bacterial isolates showing antibiotic resistance

	Antibiotics Included for Susceptibility Testing										
Species (Total #)	AMO[a]	CHL[b]	CIP[a]	CTX[a]	GEN[a]	KAN[a]	NAL[a]	STR[a]	SUL[b]	TET[b]	TMP[b]
E. coli (43)	86%	77%	93%	14%	12%	30%	91%	70%	91%	95%	79%
Enterobacteriacae (37)	84%	62%	78%	8%	8%	16%	73%	54%	84%	78%	54%
Salmonella (11)	64%	82%	73%	0%	0%	18%	73%	45%	73%	73%	64%
All Isolates (91)	82%	71%	85%	10%	9%	23%	81%	60%	86%	86%	67%

[a]Critically Important Antibiotic as defined by WHO; [b]Highly Important Antibiotic as defined by WHO; Terms used to describe the antimicrobial resistance levels are: rare, <0.1 %; very low, 0.1 % to 1 %; low, >1 % to 10 %; moderate, >10 % to 20 %; high, >20 % to 50 %; very high, >50 % to 70 %; and extremely high, >70 %.

Currently, Albania has no systems in place for the surveillance of veterinary antibiotic consumption, so it is not possible to confidently correlate usage patterns with the patterns of resistance found on in our study. There are, however, some insights worth noting that were gained through

private discussions with Albanian veterinary public health officials with practical knowledge and exposure to antibiotic usage on-farm. The first was regarding fluoroquinolone usage, which was said to be widespread and for prolonged periods on poultry farms, for therapeutic and preventive purposes, which correlates with the extremely high levels of resistance to naldixic acid (81%) and ciprofloxacin (85%) in this study's isolates. Conversely, gentamicin, kanamycin, and cefotaxime, are not known to be used in Albanian poultry, which may explain in part the low levels of resistance to the agents observed in this study. Lastly, it was indicated, while official numbers were lacking, that there was significant qualitative use, both individually and mixed, of antibiotics on Albanian farms. Further studies and data are clearly needed in the area of antibiotic consumption in Albania. Nevertheless, our observations suggest that antibiotic resistance levels reflect usage practices on poultry farms in Albania.

2.4.2 *Salmonella* and *E. coli* Isolates Exhibit Resistance to Multiple Antibiotics

Over 80% of the *Salmonella* isolates, 9 out of 11, showed resistance to at least 4 antibiotics included, and 7 isolates, over 60%, showed resistance to at least 7 of the antibiotics included, none were susceptible to all (Figure 2.2). In contrast, countries in the EU summary report did not report more than 50% of the *Salmonella* poultry isolates displaying resistance to 4 or more antibiotics, and not more than 10% displaying resistance to 7 or more [7]. Our study also found that 95% of the *E. coli* isolates, 41 of 43, showed resistance to at least 4 antibiotics, and 35 isolates, over 80%, with resistance to at least 7 antibiotics (Figure 2.2). Again in contrast, no country in the EU summary reported more than 40% of poultry *E. coli* isolates displaying resistance to 4 or more antibiotics, and none reported more than 5% with resistance to 7 or more antibiotics [7]. It is also interesting to note that high levels of multidrug resistance in other *Enterobacteriacae* suggesting there is antibiotic resistance selection and

maintenance across many bacterial species on Albanian poultry farms.

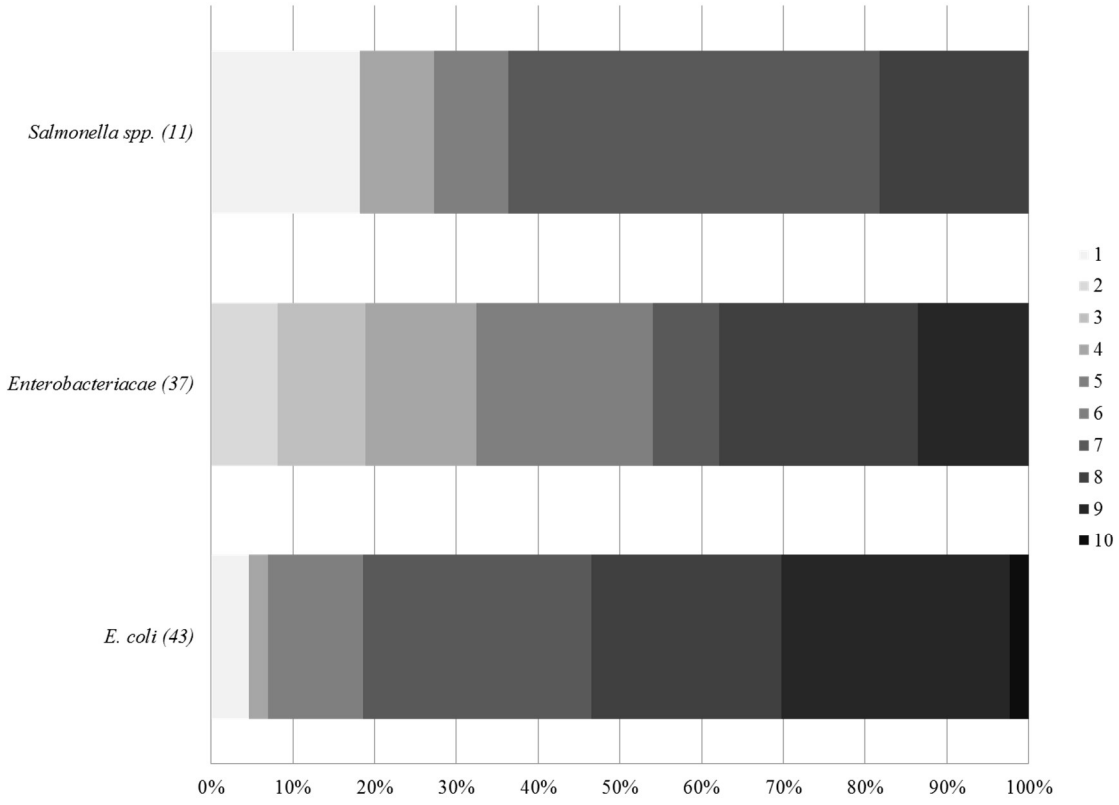


Figure 0.2 Frequency distribution of bacterial isolates resistant to one to ten antibiotics from Albanian poultry. Numbers in parenthesis indicate number of isolates.

2.4.3 Multidrug Resistant Profiles are Geographically Widespread in Albania Poultry Production

A total of 42 unique resistance patterns were identified among the isolates included. Of these, 11 patterns were either found in multiple species or across multiple farms (Figure 2.1). Profiles with multidrug resistance to the majority of antibiotics tested, like profile 5 and 17 which show resistance to 9 and 8 of the antibiotics respectively, were found on at least half of the farms sampled. The distribution of these profiles across several species in a single location, for example profile 17 in Lushnje or profile 20 in Elbasan (Figure 2.1), could be indicative of on-farm horizontal transfer of antibiotic resistance genes between species. The presence of the same resistance profile in the same species

across multiple locations, for example for profile 5 and 17 in *E. coli* (Figure 2.1), may also be indicative of clonal spread of antibiotic resistance across the country. Further genetic characterization of these isolates would provide valuable information to determine the extent of horizontal gene transfer between species and whether these resistant isolates represent geographically disperse yet clonally related populations. These molecular capabilities were not available in the lab where the pilot program's isolate characterization was performed. Further work is needed to understand the spread of these multidrug resistant strains among livestock farms, and to prevent further spread and potential transmission to the public.

2.5 Conclusion

In summary, our pilot study indicates that antibiotic resistance among bacterial isolates from poultry is much higher in Albania when compared to EU countries. Furthermore, many isolates also exhibited resistance to multiple antibiotics and are widely distributed across the poultry farms surveyed in Albania. These high levels of antibiotic resistance are significant and constitute a public health concern. Further research is needed to understand the levels of and linkages between on-farm antibiotic consumption and antibiotic resistance in Albanian, as are policies that establish systems for the monitoring of these trends and that encourage prudent antibiotic use.

2.6 Acknowledgements

This survey has been conducted as part of a joint WHO-World Organization for Animal Health (OIE) project on "National Human and Animal Health Systems Assessment Tools and Bridges", with the financial support from the European Community, represented by the Commission of the European Communities under the European Commission Avian and Human Influenza Trust Fund, World Bank acting as administrator of the grant funds (Avian and Human Influenza Facility – AHIF).

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CHAPTER 3
PHAGE & PHOSPHATASE: A NOVEL PHAGE-BASED PROBE FOR RAPID, MULTI-PLATFORM DETECTION OF BACTERIA

3.1 Abstract

Genetic engineering of bacteriophages allows for the development of rapid, highly specific, and easily manufactured probes for the detection of bacterial pathogens. A challenge for novel probes is the ease of their adoption in real world laboratories. We have engineered the bacteriophage T7, which targets *Escherichia coli*, to carry the alkaline phosphatase gene, phoA. This inclusion results in phoA overexpression following phage infection of *E. coli*. Alkaline phosphatase is commonly used in a wide range of diagnostics, and thus a signal produced by our phage-based probe could be detected using common laboratory equipment. Our work demonstrates the successful: i) modification of T7 phage to carry phoA; ii) overexpression of alkaline phosphatase in E. coli; and iii) detection of this T7-induced alkaline phosphatase activity using commercially available colorimetric and chemilumiscent methods. Furthermore, we demonstrate the application of our phage-based probe to rapidly detect low levels of bacteria and discern the antibiotic resistance of E.coli isolates. Using our bioengineered phage-based probe we were able to detect 10^3 CFU/mL of *E. coli* in 6 hours using a chemiluminescent substrate and 10^4 CFU/mL within 7.5 hours using a colorimetric substrate. We also show the application of this phage-based probe for antibiotic resistance testing. We were able to determine whether an *E. coli* isolate was resistant to ampicillin within 4.5 hours using chemiluminescent substrate and within 6 hours using a colorimetric substrate. This phage-based scheme could be readily adopted in labs without significant capital investments and can be translated to other phage-bacteria pairs for further detection.

3.2 Introduction

Pathogenic bacteria represent a significant burden on public and economic health [99-101]. The need for rapid and accurate detection of these pathogens, in both clinical and industrial settings, has spawned a large biosensor industry with a market potential over 500 million USD[102]. The search for new, innovative approaches to detection has driven research into novel probes that exploit molecular interactions, such as bacteriophage, for diagnostic use. A bacteriophage (phage), is a virus which specifically targets bacteria. Phages were first discovered in 1915 and used as antimicrobials, but were quickly supplanted in many areas of the world by the discovery and use of antibiotics[44]. The increasing prevalence of antibiotic resistant bacteria and modern development of molecular tools enabling the specific bioengineering of phages with novel functions has renewed interest in bacteriophages[55, 103]. Phage can be highly specific, they can replicate quickly, and they can be readily propagated. These traits, along with their ability to deliver genetic material to bacterial cells, make them ideal candidates for use as molecular probes for pathogen detection [54].

Phage-based diagnostics have a wide range of applications. In response to foodborne illness, and more recently the passage of the Food Safety Modernization Act, much research has focused on developing phage-based methods for the detection of low concentrations of pathogenic bacteria[50, 54]. The fruits of this research are represented commercially by phage-based diagnostics like VIDAS® UP (Biomérieux, St. Louis, MO) which uses phage components for the detection of *Salmonella*, *E. coli* O157:H7, and *Listeria*, and Sample6 which uses a phage-based bioluminescent reporter for the detection of *Listeria* in environmental samples.

In the area of antibiotic susceptibility testing, rapid DNA-based methods are increasingly supplanting traditional testing methods, but there has been little research into the actual application phage-based detection systems to this area[11, 80, 81]. While the detection range of DNA-based multiplex

systems has become quite expansive[84], there is a vast reservoir of unknown genes conferring resistance[85] and novel mechanisms of resistance being discovered regularly,[86, 87], making exhaustive testing difficult and possibly resulting in false negatives during testing of clinical isolates. There is also the possibility that genes encoding resistance are present, but not expressed, resulting in a false positive and unnecessary antibiotic treatment. Phage-based antibiotic susceptible testing avoids these issues by focusing on the phenotypic status of the cell, bacterial isolate growth in the presence of the antibiotic, rather than its genotypic status. Phage-based detection also provides several quality controls for testing. Despite the use of proficiency testing, many antibiotic susceptibility labs around the world do not have the appropriate quality control systems in place[104]. Phage host specificity means that potential cross-contamination of an antibiotic susceptible isolate's test by another laboratory isolates of a different species with resistance will not result in a positive signal as it might with a traditional or DNA–based susceptibility test. There is the possibility that a bacterial strain is resistant to the reporter phage used and not the antibiotic, resulting in a false positive. A simple test control that includes growing the isolate in the presence of the phage but without the antibiotic would catch this lack of signal due to phage resistance. These benefits make a strong case the application of phage in novel diagnostics for susceptibility testing.

Challenges for the successful adoption of new diagnostics, particularly in resource limited settings, are their cost, required maintenance, and technician training needed for their operation[105]. We propose a phage-based alkaline phosphatase probe that can be leveraged in a range of detection platforms commonly found in microbiology testing labs (Figure 3.1). This scheme would potentially reduce the need for new equipment while allowing for the rapid and sensitive detection of bacteria. Alkaline phosphatase is frequently used as an enzymatic reporter in many diagnostics, its activity can be measured using colorimetric, fluorescent, chemiluminescent, bioluminescent, and electrochemical

methods[34, 35, 106]. This allows for the potential application of our phage-based platform in widely available commercial diagnostics already optimized for alkaline phosphatase detection.

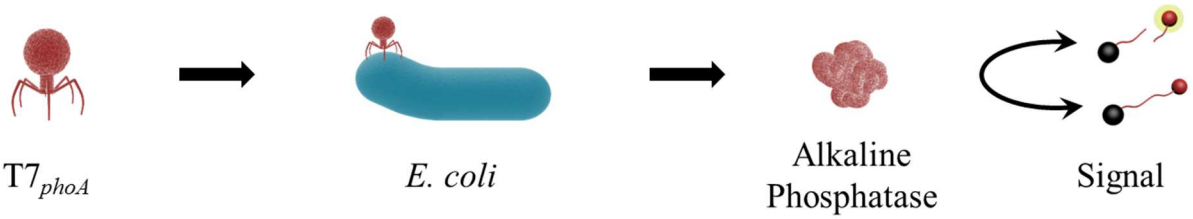

Figure 0.3 Phage-based probe. $T7_{phoA}$ is added to a sample. If viable *E. coli* are present within the sample, phage infection, replication, and alkaline phosphatase overexpression occurs. The alkaline phosphatase reporter can then be detected being using a variety of substrates.

As a proof-of-principle, we bioengineered the *E. coli* specific T7 phage [65] to carry the *E. coli* gene encoding alkaline phosphatase (*phoA*)[107]. We linked *phoA* to the T7 promoter for overexpression of alkaline phosphatase upon T7 infection of and replication within viable *E. coli*, and demonstrate the detection this T7-mediated alkaline phosphatase activity using commercially available colorimetric and chemilumiscent methods. *E. coli* is commonly used as an indicator of water quality [36], and pathogenic strains of *E. coli* are responsible for urinary tract infections[108], as well as foodborne illnessess[1]. Antibioitc resistant *E. coli* also represent a challenge when treating infections in clincal settings[109], thus novel methods that improve time-to-detection of *E. coli* are of interest. While others have used *E. coli* specific phage for either colorimetric[73] or bioluminescent [70] detection, to our knowledge we are the first to have successfully inserted a gene for alkaline phosphatase[54, 56, 103]. We demonstrate potential applications of our phage-based probe to: i) enable the detection of low concentrations of *E. coli* cells, (10^3-10^4 CFU/mL) in 6-8 hours in broth; and ii) improve time to result for antibiotic susceptibility testing of bacterial isolates. Future work will be necessary to demonstrate the robustness of this concept in more complex sample matrices, but we believe this scheme can be readily reproduced using other phage-bacteria

combinations, and could be easily adopt in many laboratories without the need for added equipment or media.

3.3 Materials and Methods

3.3.1 Bacterial Strains, Bacteriophage Strains, Media Culture, and Enumeration.

The following bacterial and bacteriophage strains were purchased from EMD Millipore (Billerica, Massachusetts): *E .coli* BL21, *E. coli* BLT5403, and bacteriophage T7Select® 415-1. Overnight cultures of both *E. coli* strains used in this study were grown at 37 °C with 200 rpm shaking in 35 mL of Luria Broth (LB), pH 7.5, contained in a 150 mL Erlenmeyer flask. Before use overnight cultures were serially diluted and plated on LB agar to confirm bacterial concentration for subsequent experiments. A double agar overlay plaque assay on LB[110] was used to enumerate phage samples. The only modification to above procures was the inclusion of 100 µg/mL of ampicillin to broth and agar for the growth of *E. coli* BLT5403 to maintain its plasmid for resistance [111].

3.3.2 Construction and Isolation of Engineered Bacteriophage

In order to modify a T7 phage that carries the *E. coli* gene for alkaline phosphatase (*phoA)* we designed a specific construct, 1743 base pairs in size, to enable T7-induced overexpression of *phoA* in *E. coli* within a pUC57 plasmid by GenScript USA Inc. (Piscataway, NJ). Our *phoA* construct was amplified with standard M13 forward and reverse primers using the iProof high-fidelity PCR kit (Bio-Rad Laboratories, Hercules, CA). All PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and digested by EcoRI and HindIII. These restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Our digested construct was then ligated into the T7 Select® 415-1 genome vector arms using T4 DNA ligase (Promega, Madison, WI) and packaged using the T7 Select® packing kits (EMD Millipore) to create $T7_{phoA}$ (Figure 3.2). We used the T7 Select® kit's packaging

control DNA, which contains the S•Tag™, as a control phage, $T7_{control}$ (Figure 3.2), which could not induce alkaline phosphatase overexpression. Following packing, $T7_{phoA}$ and $T7_{control}$ were propagated and plated as outlined by the T7 Select® kit's protocol. Individual plaques were selected, dipped into 100 µL of LB, and stored at 4 °C. All isolated plaques were PCR screened for the appropriate size insert with the T7Select® Up and Down primers using the iProof PCR kit. Plaques containing the appropriate sized insert were propagated on *E. coli* BL21, and the resulting lysates re-screened to confirm the presence of our *phoA* insert. These lysates were passed through a 0.22 µm SCFA filter (Corning Life Science, Corning, NY), tittered, and stored at 4 °C and used as our $T7_{phoA}$ and $T7_{control}$ phage stock for the further experiments.

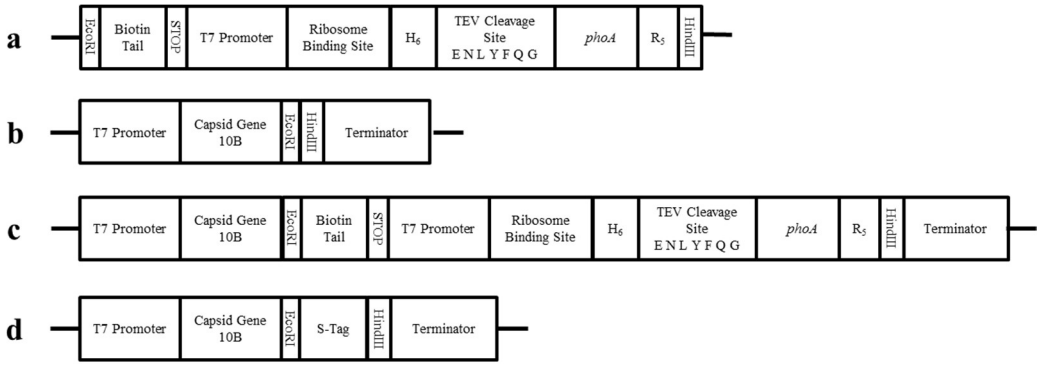

Figure 0.4 Diagram of DNA Constructs. (A) Our construct containing *phoA*. (B) Genome of T7 Select® 415-1 indicating 10B capsid protein and insertion site. (C) Genome of $T7_{phoA}$. (D) Genome of $T7_{control}$ with S•Tag™.

3.3.3 Colorimetric Detection of Alkaline Phosphatase Activity

Colorimetric detection of alkaline phosphatase activity was performed using the SensoLyte® pNPP Alkaline Phosphatase Assay Kit (AnaSpec, Inc., San Jose, CA). The sample (50 µL) was mixed with 50 µL of the kit's p-Nitrophenyl Phosphate (pNPP) colorimetric alkaline phosphatase substrate in a clear 96-well plate, incubated at 37 °C, and absorbance at 405nm read every 10 minutes, over 90 minutes,

using the Synergy 2 plate reader (BioTek Instruments, Inc., Winooski, VT). Blanks consisted of LB and pNPP substrate.

3.3.4 Chemiluminescent Detection of Alkaline Phosphatase Activity

Chemiluminescent detection was performed with two substrates. The first was alkaline phosphatase substrate Lumigen APS-5 (Lumigen, Inc., Southfield, MI). 50 µL of sample was mixed with 50 µL of APS-5 in a black 96-well plate at room temperature. Blanks consisted of LB and substrate. The plates were immediately read using a Synergy2 plate reader (BioTek Instruments, Inc., Winooski, VT) with Ex/Em filters Plug/460 nm.

The second method of chemiluminescent detection was performed using components of the Phospha-Light™ SEAP Reporter Gene Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA). A 50 µL aliquot of a 1:20 dilution of the kit's CSPD® chemiluminescent substrate in the kit's reaction buffer, which contains the Emerald™ chemiluminescence enhancer, was mixed with 50 µL of sample in a black 96-well plate at room temperature. Blanks consisted of LB and substrate. Plates were place in the Synergy2 plate reader (BioTek Instruments, Inc., Winooski, VT) for 10 minutes and read with Ex/Em filters set to Plug/Hole, respectively.

3.3.5 Confirming Alkaline Phosphatase Overexpression

Six 150 mL Erlenmeyer flasks containing 35 mL of LB were each inoculated with a 150 µL aliquot of an overnight culture of BL21. These flasks were then incubated at 37 °C for 3 hours with 200 rpm shaking, and the cultures confirmed to have reached and $O.D._{600} > 0.6$. Three cultures were then inoculated with 15 µL of $T7_{phoA}$ phage stock, and the remaining three inoculated with 15 µL of $T7_{control}$ phage stock. The cultures were incubated for 2 hours under the same conditions. The resulting lysates were then transferred to a 50 mL conical tube tubes and spun at 7598 × g on a Fiberlite F21-8x50y fixed

angle rotor (Thermo Fisher Scientific Inc., Waltham, MA) for 10 minutes at room temperature. The supernatant of each was filtered through a 0.22 µm SCFA filter (Corning Life Science, Corning, NY) and stored individually at 4 °C. All lysates were then tested for alkaline phosphates activity using both pNPP and the APS-5 substrates.

3.3.6 Bacterial Detection Using $T7_{phoA}$

Four separate overnight cultures of *E. coli* BL21 were serially diluted in LB to achieve 10^5, 10^4, 10^3 and 10^2 CFU/mL. 900 µL of each dilution and a negative control of LB were placed in 15 × 100 mm test tubes, and mixed with 100 µL of 10^3 PFU/mL of $T7_{phoA}$ in LB. Each combination, for each overnight culture, was performed in triplicate. The samples were incubated at 37 °C with 200 rpm shaking for 6 hours. Samples were then passed through a 0.22 µm SCFA filter, stored at 4 °C, and alkaline phosphatase activity was determined using all three substrates.

3.3.7 Determining Antibiotic Susceptibility of Bacterial Isolates Using $T7_{phoA}$

E. coli BL21 and BLT5403 were streaked onto the appropriate plates for single colonies. A colony of each was selected using a sterile loop and inoculated into a test tube containing 900 µL of either LB or LB containing 100 µg/mL of ampicillin. An un-inoculated test tube of LB was used as a negative control. All colony-antibiotics treatments were performed in triplicate. The tubes were then incubated at 37 °C with 250 rpm shaking for 30 minutes. Then 100 µL of 10^3 PFU/mL of $T7_{phoA}$ in LB was added to each tube. The tubes were incubated under the same conditions for 3.5 hours. For chemiluminescent detection a 50 µL samples of each tube was taken and mixed with 50 µL of the Phospha-Light™ substrate solution in a black 96-well plate and read as previously described. For colorimetric detection a 50 µL samples was mixed with 50 µL of the pNPP substrate and incubated for 1.5 hours at 37 °C in a clear 96-well plate. The plate was then read for absorbance at 405 nm using the Synergy 2 reader.

3.3.8 Statistical Analysis

Data was evaluated for statistically significance in Origin Pro version 9.0.0 (Northampton, MA). For both experiments looking at alkaline phosphatase overexpression versus a control, an unpaired one-sided t-test, assuming unknown and unequal variances, with an alpha level of 0.05 was used to test for significance. This includes the initial phosphatase expression experiment, where the average signal-to-noise ratio of all three $T7_{phoA}$ lysates was compared against that of $T7_{control}$. This was also done for the limit of detection experiment where the signal-to-noise ratio of each bacterial concentration level was compared to that of the control containing no cells. For the antibiotic resistance experiment the signal-to-noise ratio was compared between the two treatments (with/without antibiotic) within each strain type using an unpaired two-sided t-test assuming unknown and unequal variances, with an alpha level of 0.05. In all figures error bars represent one standard deviation (SD) ± from the mean and a star (*) indicates a significant difference ($p < 0.05$) between the compared sets of data.

3.4 Results and Discussion

3.4.1 Phage Construction

To construct our alkaline phosphatase reporter phage, we designed a genetic construct carrying *phoA* (Figure 3.2a) for insertion in the T7 genome (Figure 3.2b). T7 is a well-studied phage that broadly targets *E. coli* [65], and is used for phage-display [112]. Our genetic construct contains multiple components. Beginning from the 5'-terminus, we included the 1.3s biotin subunit from *Propionibacterium shermanii* transcarbonylase. The T7 Select® cloning kits enables phage-display, allowing us to fuse the 1.3s subunit to 10B capsid protein of T7[111]. The inclusion of this "biotin-tail" sequence has been shown to allow *in vivo* biotinylation of the fusion protein by *E. coli* [113]. At the 3' end the 1.3s gene we incorporated a stop codon so that our alkaline phosphatase gene would not also be fused to the capsid, but rather free in

solution. To enable T7 mediated expression of alkaline phosphatase we incorporated the T7 promoter and ribosome binding site sequences from the pET-3a plasmid (EMD Millipore). Our synthetic *phoA* sequence was initially based on the *E. coli* sequence described by Change[107]. This *phoA* gene is flanked on the 5' by a His-tag/TEV cleavage site, and on the 3' end by a 5× arginine tag. Finally we incorporated three restriction sites into our construct, EcoRI at the 5' terminus, and SalI followed by HindIII at the 3' terminus. The *phoA* coding sequence of the synthetic construct was codon optimized for expression within *E. coli* based on their proprietary algorithms. The full sequence of the construct can be found in the supplemental materials. We inserted our construct into T7 using the T7 Select®415-1 kit (Figure 3.2c) and created a T7 control using the kit's packing control DNA (Figure 3.2d). PCR was used to screen and isolate phage plaques carrying our *phoA* construct and the control.

3.4.2 Confirming ALP Overexpression

The alkaline phosphatase gene *phoA* is endemic to *E. coli*[107], and is typically expressed at low levels unless the cells are starved for phosphate[114]. To confirm that $T7_{phoA}$ exhibits increased alkaline phosphatase production over background, and not due to the stress of the phage infection itself, we compared alkaline phosphatase activity in cells infected and lysed by our $T7_{phoA}$ and our $T7_{control}$. We added an aliquot of an overnight *E.coli* BL21 culture to fresh LB and incubated it for 3 hours to ensure the cells achieved logarithmic growth. We then inoculated the cultures with either $T7_{phoA}$ or $T7_{contorl}$, allowing the phage infection and replication cycle to occur two hours before testing the resulting lysates for phosphatase activity. We tested for activity using both colorimetric pNPP (Figure 3.3a) and Lumigen-APS 5 chemiluminescent substrate (Figure 3.3b). There was a 10-fold signal-noise-ratio or greater difference in alkaline phosphatase activity between our control phage and our $T7_{phoA}$, suggesting that infection of *E. coli* by our $T7_{phoA}$ does result in alkaline phosphatase overexpression. Other research in

our lab has demonstrated the speificity of T7 phage-based detetion of *E. coli* in the presence of competitive bacterial species such as *S. enterica*, *S. aureus*, *P. aeruginosa*[115].

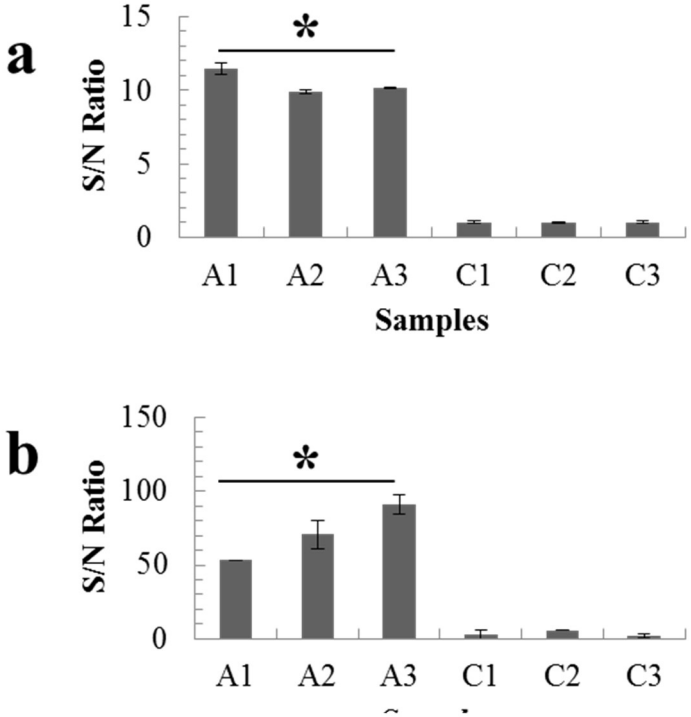

Figure 0.5 Confirming alkaline phosphatase expression. Samples A1-3 are lysates form $T7_{phoA}$, samples C1-3 are lysates from $T7_{control}$. a) alkaline phosphatase signal with pNPP substrate, b) alkaline phosphatase signal with Lumigen APS-5 chemiluminescent substrate.

3.4.3 Bacterial Cell Limit of Detection Using $T7_{phoA}$

We incubated several concentrations of *E. coli* BL21, 10^5 to 10^2 CFU/mL, and a negative control of LB, with 10^2 PFU of $T7_{phoA}$, for 6 hours at 37 °C. We then filtered the samples to remove cells and tested for alkaline phosphatase activity using three substrates: pNPP, the Phospha-Light™, and Lumigen APS-5. Significant alkaline phosphatase activity from initial bacterial levels of 10^4 CFU/mL and greater were detected using the colorimetric and chemiluminescent substrates (Figure 3.4). It should be noted

that the pNPP colorimetric assay required a 90 minute reaction time for the signal, resulting in a total assay time of 7.5 hours. For comparison, the Phospha-Light™ method only required 10 minute incubation before testing. The Lumigen APS-5 was read immediately following addition of the substrate, so does not significantly increase assay time. Furthermore, with the Lumigen substrate we were able to detect an initial cell concentration of 10^3 CFU/mL (Figure 3.4c).

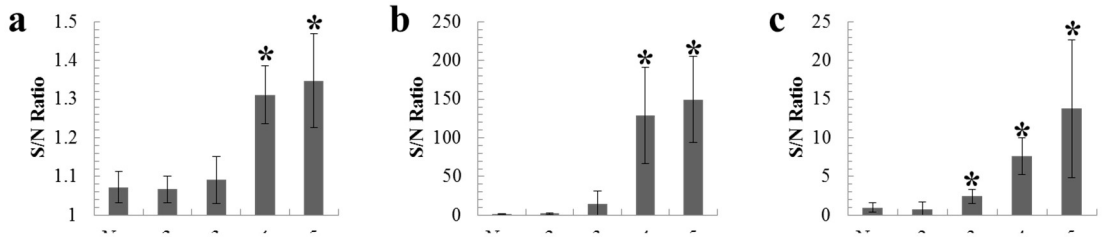

Figure 0.6 Limit of detection. Signal to Noise Ratio of alkaline phosphatase activity after 6 hour incubation of initial concentrations of *E. coli* with 10^2 PFU/mL of $T7_{phoA}$. Substrates: a) pNPP; b) PhosphaLight™; c) Lumigen APS-5.

The limit of detection (L.O.D.) of our scheme is driven by two factors. The first is the senstivity of the method used to detect alkaline phosphatase activity, as illustrated by the difference in L.O.D. of the colormetirc pNPP and the chemiluminescent Lumigen APS-5. The second factor is the total number of cells infected, which impacts the total amount of alklaine phosphatase produced. There is the potential to incorporate more sensitive detection methods for alklaine phosphatase to improve our limit of detection, for example the use of an electrochemical redoc-cycling scheme for alkaline phosphatase been shown to improve sensitivity[32]. There is also the potential to incorporate a pre-enrichment step, commonly used in when testing food samples[116], to increase bacterial levels prior to introduction of our $T7_{phoA}$. These options represent future areas of research to improve the sensitivity of this phage-based scheme.

3.4.4 Determining Antibiotic Susceptibility of *E. coli* Isolates

Traditional tests for determining the antibiotic susceptibility of a bacterial isolate do so by looking for bacterial growth in the presence of the antibiotic[117]. Broth microdilution Minimal Inhibitory Concentration (MIC) susceptibility testing in 96-well plates is a widely adopted method in clincal laboratories for assesing an isolates antibiotic susceptibility[117]. The baterial inoculum is typically prepared from isolated colonies culutred on a non-selective agar plate for 18-24 hours[117]. Several colonies, 3 to 5, are re-suspended in broth, standardized to a specific density, and then used innoculate wells containing varying concentrations of the antibiotic of interest[117]. The plates are then incubated for 16-24 hours, depending on the baterial species, and anaylzed for growth[117]. We were interested in determining if our phage-based probe had the potential to improve detection time of this later incubation step. If a bacterial isolate were resistant to a given antibiotic it should grow in broth with and without antibiotic. If we add $T7_{phoA}$, we should see phage infection, phage replication, and the production of our alkaline phosphatase reporter in both treatments. If the bacterial isolate were sensitive to a given antibiotic there will be bacterial death in the broth containing the antibiotic, resulting in no phage replication, and no alkaline phosphatase production. We should see therefore see a difference in alkaline phosphatase signal between the treatments.

For a proof-of-principle application of our modified phage, we selected colonies of *E. coli* BL21, which is susceptible to ampicillin, and colonies of BLT5403, which carries a plasmid conferring resistance to ampicillin, and used them to inoculate either broth containing 100 μg/mL of ampicillin or broth not containing the antibiotic and incubated for them for 30 minutes. $T7_{phoA}$ was then added and the samples were incubated for an additional 3.5 hours. The samples were then analyzed for alkaline phosphatase activity using pNPP and Phospha-Light™ substrates (Figure 3 .5). In our initial run, we had an overflow in pNPP absorbance readings for three samples - one of the BLT5402 colonies exposed to

ampicillin and two without - so for analysis we set the absorbance reading for those sample to be equivalent to the max read 405 nm absorbance for the run, which was 3.82. We repeated the pNPP portion of the trial, the results are an average these two trials (Figure 3.5b). For both substrates, there were no significant differences in signal between antibiotic treatments for *E. coli* strain BLT5403 strain, indicating that the strain was resistant to ampicillin. There were significant differences in signal between the treatments for *E. coli* strain BL21, indicating that the strain was sensitive to ampicillin. Total assay time was < 4.2 hours using the chemiluminescent substrate, and < 6.5 hours using pNPP. These experiments suggest there incorporation of our phage-based assay in the second incubation step of the MIC susceptibility test could reduce the 16-24 hours delay for results[117]. While the colorimetric assay takes longer than the chemiluminescent one, for resource limited labs the pNPP assay does provide the ability for visual interpretation of results as can be seen in Figure 3.3c, which is a picture of the 96-well plate from the first pNPP trial.

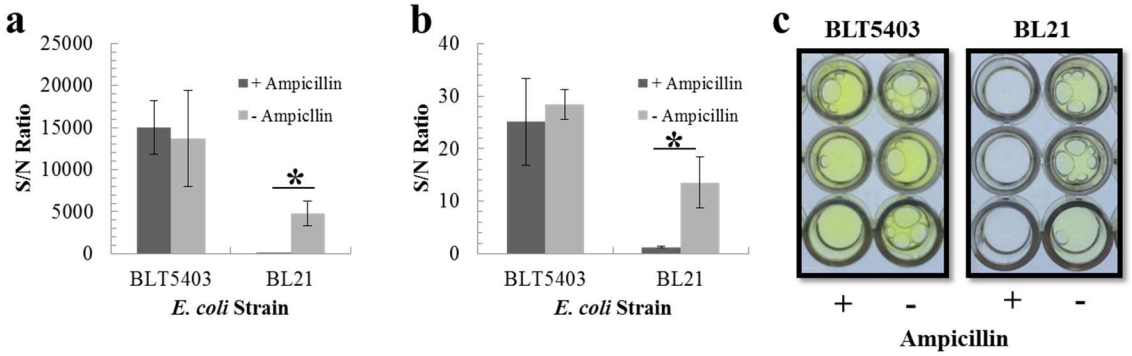

Figure 0.7 Detecting antibiotic resistance. Signal to Noise Ratio of alkaline phosphatase activity from *E. coli* stains incubated with 10^2 PFU/mL of $T7_{phoA}$ in LB with and without ampicillin. Substrates: a) PhosphaLight™; b) pNPP. c) Visual of wells with pNPP substrate.

The increasing level of antimicrobial resistance among clinical and foodborne bacteria is a major public health concern driving the need for rapid diagnostics[11]. The application of phage for the rapid detection of antimicrobial resistance is still in its infancy. MicroPhage Inc. (Longmont, CO) had

developed an FDA approved phage-based lateral flow device for the rapid detection of methicillin resistant (MRSA) *S. aureus*[82], though it is no longer commercial available[83]. One of the challenges with lateral flow devices like that of MicroPhage's, is that format does not lend itself to screening of a large number of samples. Our T7$_{phoA}$ platform is readily leverage into 96-well microtiter plates, which are commonly used in for antibiotic testing[117]. This format allows for the simultaneous analysis of a large number of samples and enables automation. We believe this platform could be readily translated to phages with specificity to other bacteria which have public health relevance, as the bioengineering of phage has been readily be demonstrated[56]. Future research standardizing our bacterial inoculum procedure and demonstrating the robustness of our phage-mediate alkaline phosphatase-based platform for antibiotic resistance testing will be needed.

3.4.5 Future Applications: Food and Water Testing

Alkaline phosphatases are ubiquitously found among bateria, animal, and plants[118] which introduces background noise for our phage-based scheme when applied to food and water samples. There a several methods which can be used to reduce this potential interferance. Bacterial alkaline phosphatases are more heat stable than those of animal and plant origin, and researchers have shown the effective use of heat treatment step to differeniate bacterial alkaline phosphatases from those of both plant[119] and animal origin[120] when testing foods and could be implemented as part of our assay.

Furthermore, we have designed several elements into T7$_{phoA}$ to address background noise in future experiments as we explore the applications of our proof-of-concept in real world testing. Our synthetic alkaline phosphatase contains both an N-terminus His-tag and C-terminus arginine tag (Figure 3.2), both of which provide ways to selectively capture our reporter enzyme, and reduce background interference from endemic phosphatases in a sample. Our phage-based construct is also designed to enable bacterial separation. We have incorporated a biotin tag fused to the capsid protein (Figure 3.2),

which allows us to attach our phage to magnetic beads coated with streptavidin, and then use these phage-magnetic beads for selective separation of *E.coli* cells from a sample. Researchers have demonstrated the parity of phage-magnetic beads to immunomagnetic separation techniques[121], and the succesfull application of these beads to selective separate a bacterial from water samples[115]. These selective elements, on the both the enzyme and phage, can potentially be used solely or in tandem to help overcome a wide range of challenges from enzymatic inhibitors to endemic phosphatases to visual inteference of signal that are common when attempting to test food and water samples.

3.5 Conclusion

Phage-based diagnostics offer powerful platforms for the rapid detection of bacteria. There has been an emphasis on using phage to detect low numbers of bacteria, but there are broader applications for phage-based diagnostics. In applications where initial bacterial loads are high, like antibiotic testing or post-primary enrichment, phage-based detection can be quite rapid and specific. In these application, phage-based diagnostic are also attractive in comparison to PCR or immunological based methods as phage are cheaply produced, specific, and as we demonstrated, readily engineered to leverage a wide range of easy-to-use, commercially available detection platforms. Another advantage to our scheme is that unlike immunological[69] and PCR-based[40] methods, phage-based detection can distinguish between viable and non-viable cells, thus lowering the incidence of false positives. More research bioengineering flexible, phage-based reporters and demonstrating multiple forms of their applications is needed to illuminate the potential of phage-based detection and ensure its successful adoption in real world diagnostics to improve public health.

3.6 Acknowledgements

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CHAPTER 4
PHAGE, PROTEASE, PEPTIDE: A NOVEL TRIFECTA ENABLING MULTIPLEX DETECTION OF VIABLE BACTERIAL PATHOGENS

4.1 Abstract

Bacteriophages represent rapid, readily targeted, and easily produced molecular probes for the detection of bacterial pathogens. Molecular biology techniques have allowed researchers to make significant advances in the bioengineering of bacteriophage to further improve speed and sensitivity of detection. Despite their host-specificity, bacteriophages have not been meaningfully leveraged in multiplex detection of bacterial pathogens. We propose a proof-of-principal phage-based scheme to enable multiplex detection. Our scheme involves bioengineering bacteriophage to carry a gene for a specific protease, which is expressed during infection of the target cell. Upon lysis, the protease is released to cleave a reporter peptide, and the signal detected. Here we demonstrate the successful: i) modification of T7 bacteriophage to carry TEV protease; ii) expression of TEV protease by *E. coli* following infection by our modified T7, an average of 2000 units of protease per phage are produced during infection; and iii) proof-of-principle detection of *E. coli* in 3 hours after a primary enrichment via TEV protease activity using a fluorescent peptide and using a designed target peptide for MALDI-TOF MS analysis. This proof-of-principle can be translated to other phage-protease-peptide combinations to enable multiplex bacterial detection, and readily adopted on multiple platforms, like MALDI-TOF MS or fluorescent readers, commonly found in labs.

4.2 Introduction

There has been a renewed interest in the application of bacteriophages (phages) for the detection and control of bacterial pathogens. In the area of diagnostics, phages are attractive as

detection elements due to their relative ease of production, host specificity, and potential for rapid signal amplification [54]. Furthermore, in comparison to immunological [69] and PCR-based [40] detection methods, phage-based detection schemes can distinguish between viable and non-viable cells, as viable cells are required for phage proliferation [122]. This potentially means that phage-based diagnostics could have lower incidences of false positives. Several phage-based diagnostics have already entered the commercial market. Biomérieux (St. Louis, MO) has a product line called VIDAS® UP which leverages phage components for the detection of *Salmonella*, *Listeria*, and *E. coli* O157. Sample6 (Cambridge, MA) uses a phage-delivered luciferase reporter for the detection of environmental *Listeria*.

Individual testing for multiple pathogens requires large sample sizes, numerous selective media and reagents, and a significant amount of time and technician labor. Multiplex methods, which use a single reaction sample to test for multiple pathogens, are attractive due to their reduced complexity and time to result. The majority of multiplex systems leverage DNA [123] or immunological [124] based methods, and thus face the potential false positive issues due to the presence of dead cells. There has been limited research in applying phage in multiplex detection schemes. Work by [125] coupled phage amplification with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (MALDI-TOF MS) for the simultaneous detection of *E. coli* and *Salmonella*, but the majority of bioengineered phage-based detection schemes have leveraged enzymatic reporters, like luciferase, that do not lend themselves readily to a single sample multiplex format [54, 103, 126].

We propose a novel phage-based platform to enable multiplex detection of bacteria using phage bioengineered to carry genes encoding highly specific proteases, whose activity is detected by the cleavage of specific peptides (Figure 4.1). The phage provides specificity for a viable bacterial target, and the protease-peptide pair provides further specificity and lays the groundwork for single sample multiplex detection. For our proof-of-principle, we genetically engineered T7, a phage with broad

specificity for *E. coli* [65], to carry a gene for a protease from tobacco etch virus (TEV). TEV protease is a highly specific protease recognizing the amino acid sequence ENLYFQ(G/S), with cleavage between the Q and G or S amino acids [127]. The cleavage event, indicative of the presence of TEV protease produced by a viable *E. coli* cells following infection with the engineered T7, can be detected using various platforms. Our work demonstrates the successful: i) modification of T7 bacteriophage to carry TEV protease gene; ii) expression of TEV protease by *E. coli* following T7 infection; and iii) detection of *E. coli* in sample via TEV protease activity. To show that this scheme was applicable to multiple platforms, we detected protease activity two ways. First, using a common fluorescent plate reader to detect the signal from a cleaved beacon peptide; and second, using MALDI-TOF MS to detect the production of a reporter peptide produced by the cleavage of a designed larger peptide. Both of these platforms are not only sensitive, but have the capability for high-throughput, multiplex screening of samples. We believe this scheme has the potential to be readily converted to other phage-protease-peptide combinations to enable multiplex detection of bacterial pathogens within a sample.

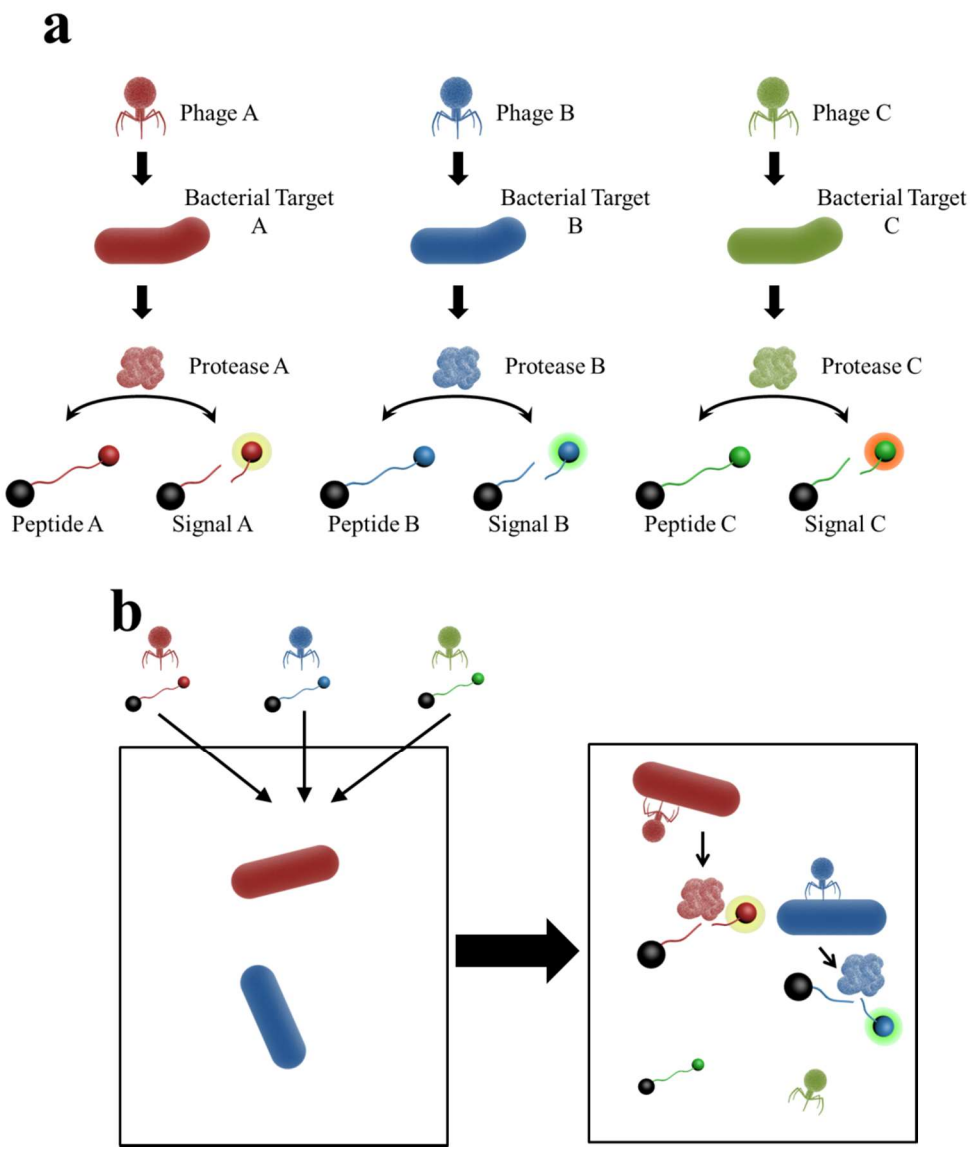

Figure 0.8 Phage-Protease-Peptide Multiplex Detection Scheme. (a) Phage specific to a bacterial target, is bioengineered to carry a gene for a specific protease. Protease acts on a specific peptide resulting in a unique fluorescent signal or peptide product. (b) Phage and corresponding peptides are added to a single sample. Signal is produced only if corresponding bacterial target is present in sample to produce the protease.

4.3 Materials and Methods

4.3.1 Bacterial Strains, Bacteriophage Strains, Media Culture, and Enumeration

Both the bacterial strain *E. coli* BL21 and the bacteriophage T7 (T7Select® 415-1) were purchased from EMD Millipore (Billerica, Massachusetts). All overnight cultures of *E. coli* BL21 were grown in 150 mL flasks containing 35 mL of Luria Broth (LB), pH 7.5, incubated at 37 °C with shaking at 200 RPM. The overnight cultures were serially diluted and spread plated on LB agar to confirm bacterial concentration for subsequent experiments. The titer of samples containing T7 bacteriophage were determined following the double agar overlay plaque assay on LB agar as commonly described [110].

4.3.2 Construction and Isolation of Engineered Bacteriophage

Using standard molecular techniques, we engineered T7 bacteriophage to carry the TEV protease gene for overexpression within *E. coli*. We designed a gene cassette (Figure 4.2) to enable T7-induced expression of TEV protease in *E. coli*, and had the cassette synthesized within a pUC57 plasmid by GenScript USA Inc. (Piscataway, NJ). The TEV protease cassette was amplified with the iProof high-fidelity PCR kit (Bio-Rad Laboratories, Hercules, CA) using standard M13 forward and reverse primers. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and then digested with restriction enzymes *Eco*RI and *Hin*dIII, both purchased from New England Biolabs (Ipswich, MA). The digested construct was then inserted into the T7 Select® 415-1 genome using T4 DNA ligase (Promega, Madison, WI) and packaged using the T7 Select® cloning kit (EMD Millipore) to create $T7_{tev}$ (Figure 4.2). In parallel, we used the T7 Select® kit's control DNA insert, which encodes for the S•Tag™, to create $T7_{control}$ (Figure 4.2), a control phage which would not induce TEV protease expression. Both $T7_{tev}$ and $T7_{control}$ packaging reactions were propagated following the T7 Select® kit's protocol and plated. Individual plaques were picked off plates using a sterile loop, dipped into 100 µL of LB, and stored at 4

°C. The isolated plaques were screened with the iProof PCR kit for the appropriate size insert using the T7Select® Up and Down primers included in the cloning kit. Isolated plaques containing the appropriate sized insert were propagated on *E. coli* BL21, and the subsequent lysates screened to confirm the presence of the insert. These lysates were passed through a 0.22 µm SCFA filter (Corning Life Science, Corning, NY), stored at 4 °C, titered, and used as the stock bacteriophage for subsequent experiments.

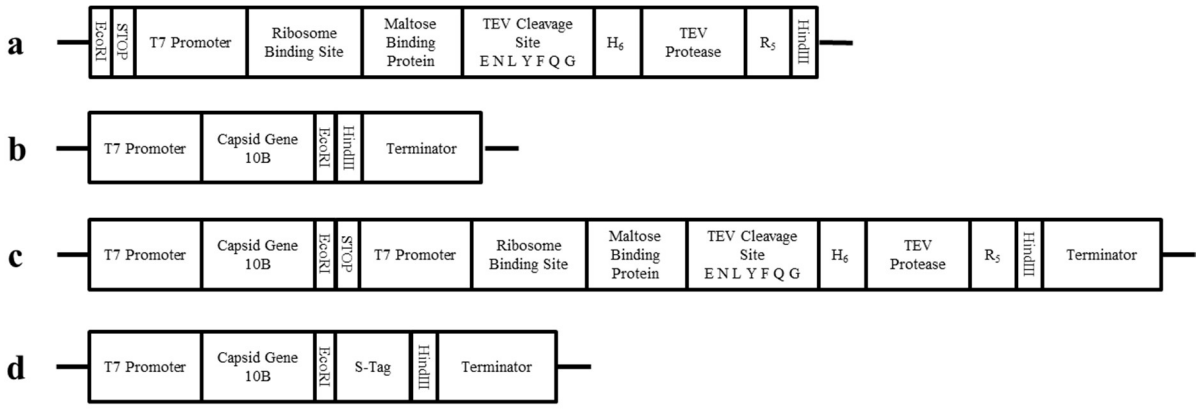

Figure 0.9 Diagram of DNA Constructs. (a) Our designed TEV protease gene cassette. (b) Genome of T7 Select® 415-1 indicating 10B capsid protein and insertion site. (c) Genome of $T7_{tev}$ with the insertion of our gene cassette. (d) Genome of $T7_{control}$ with insertion of the S•Tag™ control.

4.3.3 Fluorescent Detection and Quantification of TEV Protease

Fluorescent detection of TEV protease activity was performed using the SensoLyte® 520 TEV Activity Assay Kit (AnaSpec, Inc., San Jose, CA). 50 µL of sample was mixed with 50 µL of substrate in a 96-well plate, incubated at 30 °C, and read every 10 minutes, over 90 minutes, using the Synergy 2 plate reader (BioTek Instruments, Inc., Winooski, VT) with Ex/Em filters 485/528 nm. For quantification of TEV protease in a sample, a standard curve was run using the TEV protease standards provided with the SensoLyte® kit. Our TEV samples were in LB and the provided standards were in the kit's buffer, which meant the standards and our samples had a different base background noise. To account for this we compared V_{max}, calculated using the Gen5 software (Biotek Instruments, Inc., Winooski, VT), of the

fluorescence over 90 minutes between our samples and the standards to quantify the amount of TEV protease present.

4.3.4 Characterizing TEV Protease Production

An overnight culture of *E. coli* BL21, prepared as previously described, was serially diluted in LB to achieve 10^9, 10^8, 10^7 and 10^6 CFU/mL. 900 µL of each dilution and a negative control of LB were placed in 15 x 100 mm test tubes, and mixed with 100 µL of 10^3 PFU/mL of $T7_{tev}$ in LB. Each combination was performed in triplicate. The samples were incubated at 37 °C with 200 rpm shaking for 3 hours. Samples were then passed through a 0.22 µm SCFA filter (Corning Life Science, Corning, NY), stored at 4 °C, tittered, and TEV protease activity fluorescently determined as previously described. The experiment was repeated with two separate overnight cultures to achieve biological triplicates.

4.3.5 Target Peptide Design and MALDI-TOF Mass Spectrometry Analysis

We designed two 26 amino acid peptides, TEV-L and REF-L (Table 1), to detect TEV protease activity using MALDI-TOF MS. TEV-L was designed to contain the TEV protease cleavage site, and when cleaved by TEV protease to produce two identical 13 amino acid peptides, TEV-S (Table 4.1). The REF-L (Table 4.1) peptide was used as an internal reference. Samples of both TEV-L and REF-L were synthesized by Biopeptide Co., Inc. (San Diegeo, CA), at 98% purity. Samples were reconstituted in sterile water to make a 1 mM stock solution.

Reagents for MALDI-TOF MS analysis are as follows: triflouroacetic acid (TFA), α -cyano-hydroxycinnamic acid (α-CHCA), and Tetrahydrofuran (THF). TFA and α-CHCA were purchased from Sigma-Aldrich (St. Louis, MO), and THF was purchased from Fisher Scientific (Pittsburgh, PA). For MALDI-TOF MS analysis, 5 µL of a matrix solution (0.16 M α-CHCA in 69: 30: 1% THF/H_2O/TFA) was mixed with 5 µL of a sample. 2 µL of the matrix/sample mixture was then spotted onto a stainless steel target and the

solvent was allowed to evaporate at room temperature. Analysis was performed using a Bruker Autoflex III smartbeam time-of-flight mass spectrometer (Bruker Daltonics, Inc., Billerica, MA). All spectra presented were obtained in reflectron mode and represent an average of 5000 shots acquired at 46% laser power.

Table 0.2 Peptides Deigned for MALDI-TOF detection of TEV Protease Activity

Name	Amino Acid Sequence	Residue Number	Molecular Weight
TEV-L	SHFLKKR**ENLYFQ#S**HFLKKRENLYFQ[1]	26	3401.93 g/mol
TEV-S	SHFLKKRENLYFQ	13	1709.95 g/mol
REF-L	GPHFLKKR**ETLFQ#GP**HFLKKRETLFQ[2]	26	3183.72 g/mol
REF-S	GPHFLKKRETLFQ	13	1600.87 g/mol

[1]Bold letters designate the recognition sequence for TEV Protease, the hashtag indicates the cleavage site. [2]Bold letters designate the recognition sequence for HRV-3C Protease, the hashtag indicates the cleavage site

4.3.6 Detection of *E. coli* Through TEV Protease Activity Using Fluorescence and MALDI-TOF MS

Three colonies of *E. coli* BL21 streaked out onto an LB agar plate were selected, each put into 35 mL of LB in a 150 mL flask, and incubated at 37 °C with shaking at 200 rpm for 16 hours to simulate a primary enrichment. 150 µL of this primary enrichment was added to 35 mL of fresh LB in a 150 mL flask and incubated for 3 hours under the same conditions. Then 10 µL of $T7_{tev}$ phage stock (1.08×10^{11} PFU/mL) was added and the sample incubated for another two hours under the same conditions. LB inoculated with 10 µL of $T7_{tev}$ stock was used as a control. The resulting lysates were then filtered with a 0.22 µM filter to remove any cellular debris. Each lysate was analyzed in triplicate for TEV protease activity using the SensoLyte® fluorescent assay as previously described. For MALDI-TOF MS analysis 12.5 µL of each lysate was mixed with 12.5 µL of solution containing 10 µM of TEV-L and REF-L peptides in TEV reaction buffer from the SensoLyte® kit. Samples were incubated for 1 hour at 30 °C, heated to 80 °C for 10 minutes to deactivate any TEV present, and then cooled to 4 °C until MALDI-TOF MS analysis.

4.4 Results

4.4.1 Constructing a TEV Protease Producing Bacteriophage

To create our proof-of-principle phage-based enzymatic multiplex detection of bacteria, we designed a gene cassette containing a gene for TEV protease (Figure 4.2a) for insertion in the T7 genome (Figure 4.2b), a phage with broad host range specificity for *E. coli* [65] We built upon a TEV protease mutant S219V which was shown to be more stable and catalytically efficient than the wild type when expressed in *E. coli* [127]. The S219V TEV protease gene is linked to a maltose binding protein (MBP) at the N-terminus by a short amino acid sequence containing the TEV cleavage site sequence, ENLYFQG, followed by a 6X His-tag. At the C-terminus there is a 5X arginine tag. The MBP chaperone and ability of the protease to self-cleave from the chaperone was shown to increase solubility and activity of the protease when expressed in *E. coli* [128] Upstream of the start codon for MBP, we added the T7 promoter sequence and ribosome binding site sequence from the pET-3a plasmid (EMD Millipore), to allow for T7-mediated overexpression of the protease. The T7 Select® cloning kits are designed to enable phage-display, and the inserted peptide sequence is fused to 10B capsid protein of T7 [111] We wanted the protease to be free in solution, not attached to the phage capsid, so we followed the example of [129], and added a stop codon in all three reading frames upstream of our T7 promoter. Lastly, we added the restriction site for *Eco*RI at the 5' terminus of our cassette and the *Hin*dIII site at the 3' terminus. We removed a *Hin*dIII site found within the TEV protease gene. Our gene cassette was synthesized by Genscript, Inc., and the codon sequences optimized using their proprietary OptimumGene™ algorithm for expression within *E. coli*. The full sequence of our cassette can be found in via Genbank, accession number KT183030. We cloned our cassette into the T7 Select®415-1 genome (Figure 4.2c) and as a control cloned the T7 Select® kit's control insert, encoding the S•Tag™ (Figure 4.2d).

After cloning both inserts, we used PCR to screen plaques for phage that contained either our gene cassette or the control insert. We choose two plaques, 15 and 41, carrying our gene cassette and one plaque carrying the control insert, to look for TEV protease activity. We propagated the plaques in a broth of *E. coli*, and after incubation filtered the lysates to remove any remaining cells. We took samples of each of the three lysates and tested for TEV protease activity using the SensoLyte® fluorescent TEV assay. We monitored fluorescence over 90 minutes, and divided the signal by the signal of a known negative control, a sample containing only LB and the fluorescent peptide substrate (Figure 4.3). After 90 minutes, we could clearly observe an increase in fluorescence vs. the negative control in both phage samples carrying the TEV gene cassette and did not observe an increase in fluorescence in the phage sample carrying the control insert. We thus show that not only have we successfully introduced our TEV protease gene cassette in T7, but that the protease is produced in an active form by *E. coli* following infection by our $T7_{tev}$. For all further experiments, we used $T7_{tev}$ phage derived from our plaque 15 lysate.

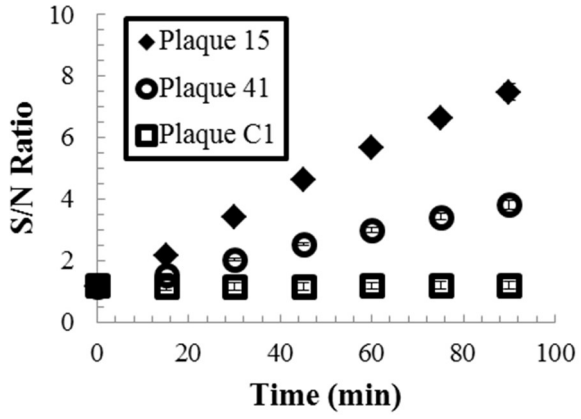

Figure 0.10 Confirming TEV protease product. Ratio of fluorescent signal in lysate samples to that in an LB negative control over time. Plaque 15 and 41 are phage confirmed by PCR to carry the TEV protease gene cassette, Plaque C1 carries the S•Tag™ insert.

4.4.2 Characterizing TEV Protease Production

In order to understand how much TEV protease was being produced and how many *E. coli* cells were needed to obtain a signal, we incubated several concentrations of *E. coli* BLS21, 10^9 to 10^6 cfu/mL, and a negative control of LB, with 10^2 PFU of $T7_{tev}$, for three hours at 37 °C. We then filtered the samples and monitored fluorescence over 90 minutes, and normalized the signal to the negative control (Figure 4.4). We observed an increase in fluorescence vs. the negative control after 90 minutes in samples with a starting CFU/mL of 10^7 and 10^8, but observed no increase in the others.

Using the TEV protease standards provided with SensoLyte® assay we determined the amount of protease produced in the 10^7 and 10^8 CFU/mL samples to be 400 ± 300 ng of TEV/mL and 1500 ± 500 ng of TEV/mL, respectively (data not shown). TEV protease is a 25 kDa protein [127], and using its molecular weight we can calculate the number of TEV protease molecules present per mL in these two

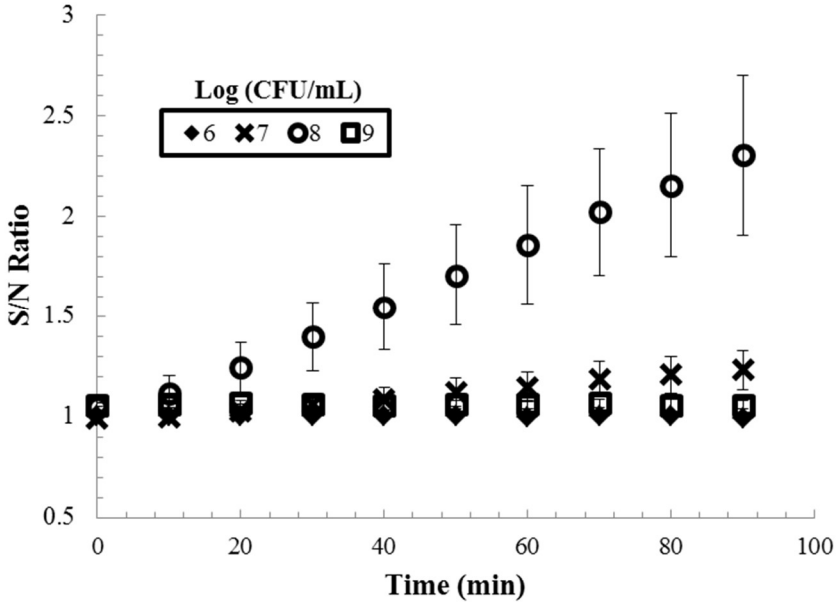

Figure 0.11 TEV protease activity from varying starting cell concentrations. Ratio of fluorescent signal in $T7_{tev}$ lysates (with starting cell concentrations of 10^6, 10^7, 10^8, and 10^9 CFU/mL) to that of a control with no cells, over time.

samples to be $10^{13.0}$ and $10^{13.6}$, respectively. The final phage titer of the 10^7 and 10^8 samples was determined to be $10^{9.86 \pm .06}$ and $10^{10.17 \pm .13}$ PFU/mL, respectively. Dividing the average number of molecules of TEV protease produced by the average amount of phage produced, results in approximate values of 1380 and 2754 in the 10^7 and 10^8 samples, indicating that roughly 2000 molecules of TEV protease are produced for every phage produced by an infected *E. coli* BL21 cell.

4.4.3 Designing Peptides for MALDI-TOF

MALDI-TOF mass spectrometry can detect small peptides with a relative molecular mass of ~1000-3000 at sample concentrations as low as 1 nM, though as the complexity of the sample matrix increases the sensitivity typically decreases to > 1 µM of target peptide [130]. We designed two peptides, TEV-L and REF-L (Table 4.1), for detection by MALDI-TOF MS. TEV-L was designed so that when cleaved by TEV protease it would produce two identical peptides, TEV-S (Table 4.1). This provided us with two advantages: first, a doubling in signal would be expected as for every cleavage of TEV-L we would get two TEV-S peptides; and second, increased specificity would be present since cleavage of TEV-L by a protease other than TEV would likely not result in the production of two identical smaller peptides.

4.4.4 Detection of *E. coli* Through TEV Protease Activity Using Fluorescence and MALDI-TOF

For a proof-of-principle, we used a culture of *E. coli* BL21grown from a single colony for 16 hours in LB as a stand in for a non-specific primary enrichment typically used for the detection of food borne pathogens, like *E. coli* O157:H7 and *Salmonella spp.* [131]. We took a portion of this primary enrichment and inoculated fresh LB, incubated for 3 hours, then added our $T7_{tev}$, and incubated another 2 hours. The samples were filtered to remove any remaining bacterial cells, and then analyzed for TEV protease activity using the fluorescent kit (Figure 4.5) and MALDI-TOF MS (Figure 4.6). Both methods were able to detect TEV protease activity in the samples containing *E. coli*. In the majority of the *E. coli* positive samples, MALDI-TOF MS analysis could not detect any remaining TEV-L, whereas a TEV-S peak was always observed.

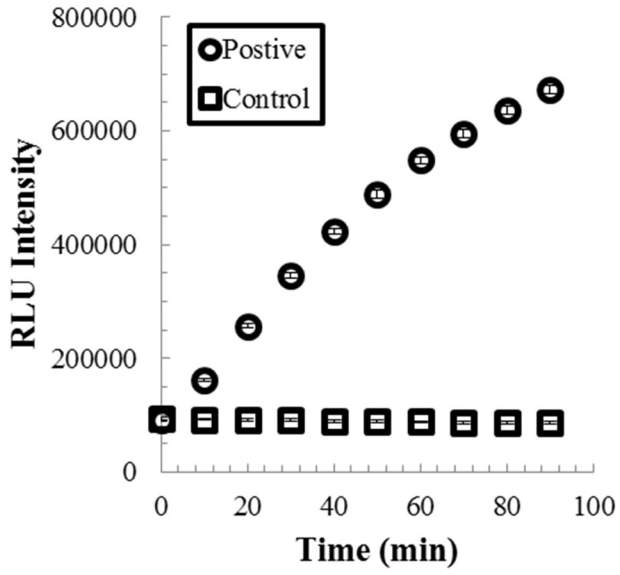

Figure 0.12 Fluorescent detection of *E. coli* via T7-induced TEV protease activity. Fluorescent signal intensity of *E.coli* positive samples and a negative (Control) over time.

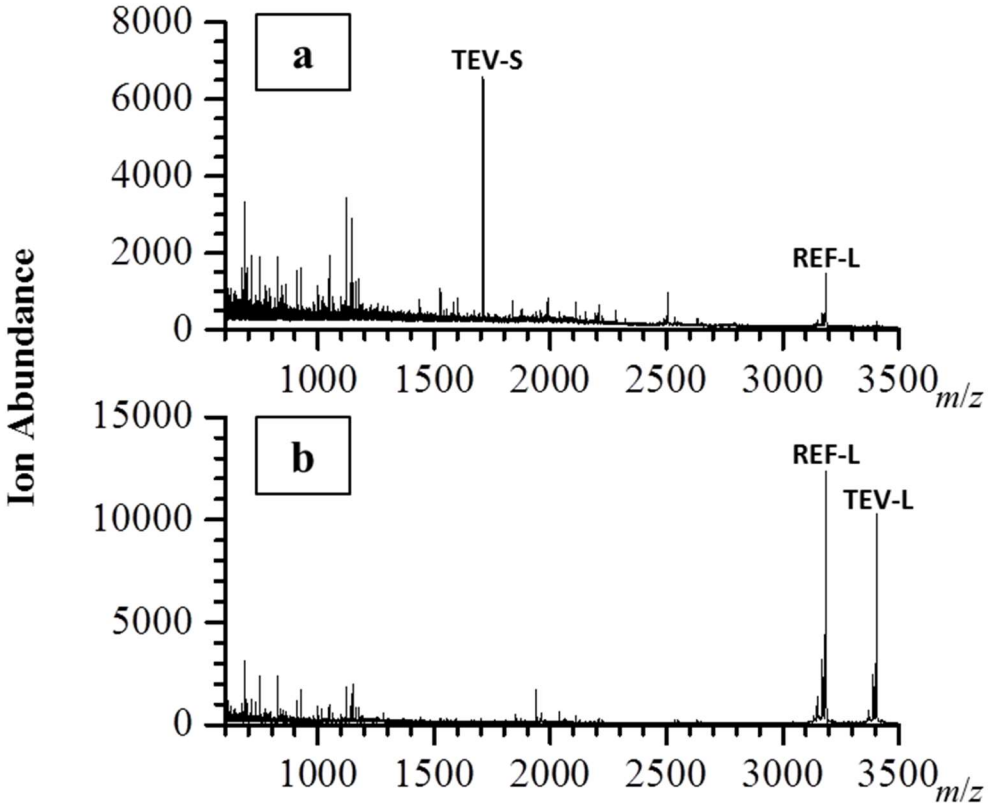

Figure 0.13 MALDI-TOF detection of *E. coli* via T7-induced TEV protease activity. Spectra of representative *E.coli* positive sample (a) and negative control (b). The peaks corresponding to TEV-L, TEV-S, and REF-L are labeled.

4.5 Discussion

In this work we demonstrate the production of a highly specific protease delivered by an engineered phage for the detection of *E. coli* using fluorescent and MALDI-TOF MS platforms. While the bioengineering of reporter phage is not new [54, 56, 103], to our knowledge we are the first to demonstrate the use of a protease as a reporter with application in multiple detection platforms. While our proof-of-principle is specific for *E. coli*, we believe our scheme can be readily modified for the detection of other bacterial species in combination with other specific proteases. Successful bioengineering of phage specific for other species, like *Salmonella* and *Listeria*, has been well demonstrated [56]. The successful

expression of viral proteases in gram negative bacteria, like E. *coli*, has also been demonstrated [132-134]. This phage-protease-peptide combination lays the groundwork for a novel multiplex detection scheme of various bacteria within a sample.

Of the two platforms we leveraged for the detection of *E. coli* via phage-induced TEV protease activity, fluorescence is likely the most widely adoptable. Fluorescent/luminescent readers, both high-throughput, like Synergy™ 2 (BioTek) or GloMax® (Promega), and smaller mobile readers, like the Quantus™ Fluorometer (Promega) are commercially available and commonly used. There is also a wide range of fluorescent/quencher markers for substrate-based protease probes [135, 136] suggesting that a mature phage-protease-fluorescent peptide multiplex scheme could be easily implemented by many laboratories.

The upfront equipment cost necessary for a MALDI-TOF MS system may be higher than that of a fluorescent system, but the cost is lowering to that point that when combined with the low cost and high speed of sample analysis, MALDI-TOF systems are beginning to be adopted in high and low resource countries [137-139]. MALDI-TOF MS analysis offers several advantages for our detection scheme. The first is the higher resolving power of MALDI-TOF MS over fluorescence. MALDI-TOF MS differentiates small peptides based on their mass-to-charge (m/z) ratios, and they need only differ by a couple m/z units to be easily distinguished. One could readily design a very wide range of peptides for detection, and thus the breadth of the multiplexing capability is much greater with MALDI-TOF MS. To expand the breadth of multiplexing with a fluorescent readout, one would need to incorporate more and more fluorescent molecules whose emission spectra may not be resolved well. The second advantage MALDI-TOF MS offers is specificity. There is the possibility that a given sample may contain an extraneous protease that cleaves our peptide, producing a signal in our fluorescent scheme, resulting in a false positive. The TEV-L peptide is designed to be cleaved into two identical peptides, TEV-S, by TEV

protease. It is unlikely that the activity of another protease would result in peptides whose m/z ratio would be identical to TEV-S. Thus, using the MALDI-TOF MS platform we can be further assured that the signal we measure is only due to TEV protease activity, which would only be present if viable *E. coli*, infected by $T7_{tev}$, were present in the original sample. A third advantage to the MALDI-TOF MS platform is the signal amplification inherent in our scheme, where TEV protease cleavage of the TEV-L peptide results in two TEV-S peptides. This can be observed by comparing the signal TEV-L to REF-L in the control to the signal of TEV-S to REF-L in the *E. coli* positive samples (Figure 4.6). There is the potential that this signal amplification and the sensitivity of the MALDI-TOF MS could improve the bacterial limit of detection beyond that of the fluorescent scheme, which is approximately 10^7 CFU/mL (Figure 4.4). Further work determining the limit of detection for the peptides, optimizing the sample matrix for both TEV activity and background signal, and the use of sample enrichment tools that can be readily coupled with MALDI, like Surface-Enhanced Laser Desorption/Ionization [140], need to be explored before understanding if this benefit can be realized.

PCR-based detections methods have clearly demonstrated their sensitivity, though DNA contamination remains a challenge. Coupling PCR with a sample pre-treatment of ethidium bromide monoazide or propidium monoazide, dyes that irreversible bind DNA, has been shown to enable PCR-based differentiation of viable and non-viable cells [141-143]. Studies, however, suggest more research is needed to optimize the successful use of these dyes. When used at low concentrations, interfering compounds or high levels of non-viable cells, both of which may be found in environmental or foods samples, can still result in false positive [144, 145]. At high concentrations, these dyes are toxic, expensive, and have been shown to have a negative effect on the detection of DNA from viable cells of clinical important pathogens [145-147]. It therefore seems prudent to further probe the capabilities and potential applications of phage-based detection of viable bacterial cells.

A challenge for phage-based bacterial detection is the condition of the target cell. Environmental conditions, temperature, bacterial growth rate, membrane composition, and cell injury are all factors that have been shown to impact the success of phage infection and subsequent productivity of infection [122]. This is a key reason we focused on bacterial detection following primary enrichment, to ensure the cells were in a state favorable for infection. In fact, when we looked for TEV activity in samples containing 10^9 CFU/mL, we could detect none (Figure 4.4). This likely occurs because at a concentration of 10^9 CFU/mL the majority of *E. coli* cells are in stationary rather than log phase, thus significantly limiting cell growth and phage replication. To address this upper limit, we added a step where a portion of the primary enrichment sample was added to fresh both and incubated to allow the cells to re-enter log phase before the addition of phage. Another reason for a primary enrichment is that the level of a pathogenic bacteria in a sample like food can be quite low, and the sample sizes needed to detect their presence are large enough, 25 g to 375 g [131], to make detection without an enrichment step unfeasible. A study by [116] found that injured pathogenic bacteria only grew to levels between $10^{4.0}$ to $10^{8.3}$ CFU/mL when revived in pre-enrichment broth after 24 hours. A study by [148], using SEL broth [149], found that injured pathogenic cells of *E. coli*, *Salmonella*, and *Listeria* reached levels > 10^9 CFU/mL after a 20 hour enrichment. These studies further highlight the need for the appropriate enrichment conditions to ensure bacterial and successful detection. In future work we intended to implement a concentration step, either filtration based or using phage-based magnetic bead separation [115] following appropriate primary enrichment conditions to ensure that necessary cell concentrations are consistently achieved.

4.6 Conclusion

In summary, bioengineering of bacteriophage offers novel platforms for the specific and sensitive multiplex detection of bacterial pathogens within a sample. By utilizing molecular reporters

with multiple modes of detection, one can adapt a base technology, in this case phage-based enzymatic reporters, to meet the varying capabilities of laboratories around the world. To further explore this multi-platform approach to detection, our lab is also designing a paper-fluidic, immuno-lateral flow assay for maltose binding protein, a by-product of our T7-mediated overexpression of TEV protease, as an alternative detection method for the presence of *E.* col that can be performed in resource-limited environments, like on-farm. Continued research and novel phage constructs will further expand the application and use of phage-based diagnostics for bacterial detection in the real world.

4.7 Acknowledgements

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CHAPTER 5
BIOENGINEERING BACTERIOPHAGE TO ENHANCE SENSITIVITY OF PHAGE AMPLIFICATION-BASED PAPER FLUIDIC DETECTION OF BACTERIA

5.1 Abstract

Bacteriophage (phage) amplification is an attractive method for bacterial detection due to phage-host specificity, short amplification time, and its ability to differentiate between viable and non-viable cells. Modern molecular techniques have further enabled the bioengineering of phages to co-produce reporter molecules, such as peptides and enzymes, thus increasing the sensitivity of phage amplification-based detection. The outcome of these advances is an array of targeted bacterial detection probes that are easily produced. The next step is leveraging bioengineered phage to create cheaper, faster, and easier to use detection platforms. Paper fluidic devices, like the lateral flow assay, are ideal testing platforms due to their low cost of production, simplicity, portability, and reliability. Our work investigates the use of bioengineered T7 phage strains to increase the sensitivity of phage amplification-based lateral flow assays. In this work we demonstrate a greater than 10-fold increase in sensitivity using a phage-based peptide reporter, maltose-binding protein, over the detection of T7 phage virion itself, and a greater then 100-fold increase in sensitivity using the a phage-based enzymatic reporter, alkaline phosphatase. This increase in sensitivity enabled us to detect 10^3 CFU/mL of *E. coli* in broth after 7 hours, and by adding a filter concentration step, the ability to detect a regulatory relevant *E. coli* concentration of 100 CFU/100 mL in artificially contaminated river water after 9 hours. The combination of the paper fluidic format with phage-based detection provides a platform for the development of novel bacterial diagnostics that are sensitive, rapid, and simple to use.

5.2 Introduction

The application of bacteriophage (phage) as detection probes has many advantages. Phages are relatively ease to produce, they are host specific, can potential distinguish between viable and non-viable cells, and their use allows for rapid signal amplification[54]. The use of phages for signal amplification is commonly referred to as phage amplification. In concept, a single phage adsorbs to and infects a single bacterial cell. The phage DNA is injected into the host, and through a series of mechanisms, it essentially high-jacks the metabolic machinery of the cell into the production of new phage. At the appropriate point in the infection cycle lysozyme is produced, rupturing the cell, and releasing tens to thousands of new phage into the environment, an increase that can be detected through traditional plating methods such as a plaque assay or other detections scheme like ELISA. For a phage such as T7, which has broad range specificity for *E. coli,* time from infection to lysis occurs in as little as 25 minutes[65], after which over 100 infectious particles (i.e. new phages) are released[66]. Therefore, in the time it would take an *E. coli* cell to double[67], there is a potential 100-fold increase in T7 phage, making it an attractive component in a detection scheme for *E. coli*.

Advances in bioengineering have allowed the genetic modification of phages to co-express reporter peptides and enzymes during host cell infection, further enhancing the magnitude and speed of signal amplification[54, 70, 73, 75]. Both modified and unmodified phage amplification schemes have been leveraged in bacterial detection methods[126], but little research has attempted to combine phage amplification with a resource-limited amenable platform, such as lateral flow assays (LFA). Paper-fluidic[16, 17] and microfluidic[18, 19] based devices, like LFAs, are attractive testing platforms due to their ease-of-use, cost, and reliability. They have been shown to be effective platforms for the detection of analytes and pathogens of interest in low-resource

settings[14]. The simplicity and ubiquity of the home pregnancy test highlights the attractiveness of the LFA, particularly when testing liquid matrices.

LFAs for the detection of pathogenic strains of *Escherichia coli* have previously been investigated[22, 23], and there are commercially available products for some *E. coli* strains such as the DuPont™ Lateral Flow System for *E. coli* O157:H7 (DuPont, Wilmington, DE) and Neogen's Reveal 2.0 for *E. coli* O157:H7 (Neogen, Ann Harbor, MI). The challenge with these immuno-based lateral flow assays is the difficulty in developing a mixture of antibodies with a broad enough range to capture an entire species of bacteria, such as the generic *E. coli*, which is commonly used as indicator of water quality [36], and a marker of urinary tract infections[150]. These LFAs also require a heat inactivation step, indicating that these assays actually detect non-viable rather than viable cells. If the original sample has high levels of heat-killed cells, perhaps due to a validated pathogen reduction step, the test could result in a false positive. Furthermore, many of these LFAs require an enrichment of 8-24 hours prior to testing. Phages offer potential solutions to challenges. Phage strain selection offers the ability to narrow or broaden the bacterial specificity of the test. Phage can only amplify in the presence of viable cells, thus reducing the potential of false positives, and the dynamics of phage amplification could conceivably enhance detection time.

In previous work, our lab bioengineered two reporter T7 phage strains: i) $T7_{TEV}$ which carries a gene for tobacco etch virus (TEV) protease that is expressed upon infection of *E. coli* [151]; and ii) $T7_{ALP}$, which carries the *E. coli* alkaline phosphatase gene that is overexpressed during infection[152]. A co-product of $T7_{TEV}$ infection is maltose-binding protein (MBP), which acts as a chaperon for the folding of TEV protease before being cleaved from the construct[128]. Approximately 2000 units of TEV protease, and concurrently molecules of MBP, are produced

per phage during infection[151]. Given that T7 has a burst size of ~100 PFU per cell, there is a potential production of 200,000 MBP molecules per bacterial host. MBP is also used as a protein capture tag in purification schemes, so commercial antibodies are widely available, making the construction of an LFA for MBP quite pragmatic. Alkaline phosphate is a commonly used enzymatic reporter[118], with readily available commercial antibodies against it as well. Furthermore, when paired with a colorimetric substrate like 5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium (BCIP/NBT), alkaline phosphatase can act as its own reporter, thus eliminating the need for a secondary reporter antibody and simplifying the standard sandwich LFA scheme. Both bioengineered T7 reporters: MBP due to its level of expression per cell; and alkaline phosphatase due to its enzymatic activity; make attractive targets for an LFA with the potential to improve sensitivity in a phage amplification-based detection scheme.

Our study is composed of three parts. The first investigates the dynamics between initial T7 phage inoculum concentrations and *E. coli* concentrations on subsequent phage amplification over time, providing us with an understanding of the level of phage amplification we can reasonable expect from a given starting concentration of cells. The second part focuses on whether bioengineered phage can enhance the sensitivity of phage amplification-based LFAs. We develop and compare three LFAs targeting different reporter products from phage-amplification schemes: i) the wild-type T7 phage virion itself; ii) a MBP reporter produced by a bioengineered T7 phage; and iii) an alkaline phosphatase reporter that is delivered by a bioengineered T7 phage. Lastly, we investigate whether or not an increase in LFA sensitivity translates into increased sensitivity for bacterial detection. To do this we demonstrate a proof-of-principle application of our phage amplification-based lateral flow assay scheme for the

detection of low levels of *E. coli* in media and river water. We demonstrate the ability of bioengineered phage-based reporters to improve the sensitivity of phage amplification-based LFA detection schemes several orders of magnitude, thus enabling the rapid detection of a low bacterial concentrations in artificially contaminated broth and river water. Our research highlights the potential of bioengineered phage-based reporters in LFAs targeted to bacterial, and we believe further research in this area can lead to the development of rapid, sensitive, and cheap diagnostics assays for bacterial detection.

5.3 Materials and Methods

5.3.1 Bacteriophage Strains, Working Stock Preparation

E. coli BL21 stock was purchased from EMD Millipore (Billerica, Massachusetts) and wild-type bacteriophage T7 stock was purchased from ATCC (BAA-1025-B2, Manassas, Virginia). *E. coli* BL21 culture was routinely grown overnight in 50 mL of Luria Broth (LB), pH 7.5, at 37 °C with 250 rpm shaking. An overnight culture (1 mL) was then added to 200 mL of fresh LB and incubated at 37 °C with shaking until an optical density (OD) of at least 0.6 at 600 nm was reached, typically after 3.5 hours. Then 20 µL of T7 stock was added to the *E. coli* BL21 culture and allowed to incubate, with shaking, at 37 °C for 1.5 hours. NaCl (5g) was added to the 200 mL culture to prevent further phage adsorption to the cells. The culture was then divided into six 35 mL tubes and spun at 7598 X g on a Fiberlite F21-8x50y fixed angle rotor (ThermoFisher Scientific, Waltham, MA) for 10 minutes at room temperature. Supernatant was collected and filtered through 0.22 µm SCFA filter (Corning Life Science, Corning, NY). 40 mL of this filtered culture was divided into four 13.5 mL ultracentrifuge tubes and spun at 105644 X g for 2 hours on a Fiberlite F65L-6X13.5 fixed angle rotor (ThermoFisher Scientific) at room temperature. The

supernatant was removed and pellets in each tube were re-suspended in 1 mL of 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, and then combined for a total of 4 mL of purified T7 working stock. Working stock of Phage $T7_{TEV}$, which causes *E. coli* to overexpress tobacco etch virus protease and maltose binding protein upon infection, and working stock of Phage $T7_{ALP}$ which causes *E. coli* to overexpress alkaline phosphatase upon infection, were prepared as previously described [151, 152]. For the sake of clarity, $T7_{TEV}$ will be referred to a $T7_{MBP}$, as we are interested in MBP production for this application. The titer of all T7 working stocks were determined following the double agar overlay plaque assay[110].

5.3.2 Phage Concentration Versus Cell Concentration Matrix

For each matrix an overnight 50 mL culture of BL21, was grown at 37 °C in LB broth with shaking. OD was determined to confirm sufficient growth had occurred to achieve a concentration of approximately 10^9 CFU/mL. The overnight culture was serially diluted down to 10^{-6} (~1000 CFU/mL) 10^{-7} (~100 CFU/mL), 10^{-8} (~10 CFU/mL), 10^{-9} (~1 CFU/mL) using LB broth. 1 mL of the desired dilution was pipetted into the appropriate well in the 96-well DeepWell™ (ThermoFisher Scientific) plates. In case where the initial CFU/mL desired was 0, 1 mL of sterile LB broth was added to the well. Each dilution series was plated on *E. coli*/coliform petrifilm (3M, St. Paul, MN) or spread plated on LB agar to confirm bacterial levels.

The T7 working stock, which in all cases was in the 10^{11} PFU/mL range, was serially diluted down to desired levels (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) in LB broth and 10 µL of the appropriate dilution was pipetted into the appropriate well to achieve an initial well PFU/mL of 10^4, 10^3, 10^2, and 10^1 respectively. In cases where the desired initial PFU/mL was 0, 10 µL of sterile LB broth was added to the well.

The 96-well plate was then incubated at 37 °C with shaking at 250 rpm for either 3, 5, or 8 hours. At the end of the desired incubation times, 100 µL of 5 M NaCl was added to each well to stop phage adsorption, and the contents of each well was passed through a 4mm 0.45 µm Millex®-HV filter (EMD Millipore) to remove any cells and cellular debris, and placed in the corresponding micro-centrifuge tubes and stored at 4 °C. Each tube was then tittered following the double overlay method to determine final PFU/mL. All plates and time points were performed in triplicate.

5.3.3 Lateral Flow Assay Construction

T7 lateral flow strips were constructed as follows. A 10 cm by 2.5 cm strip of nitrocellulose membrane (AE 98, Whatman International, Kent, England) was backed with a 10 cm by 4 cm strip of PET plastic using 3M 465 adhesive transfer tape. For the test line, a solution of T7 tail fiber monoclonal antibody (EMD Millipore), which had been dialyzed overnight against PBS buffer pH 7.2, was printed across the backed membrane in a 95 mm line to achieve a concentration of ~0.2 µg of antibody per mm using the Linomat IV (Camag Scientific, Inc., Willmington, NC). The membranes were placed in a vacuum oven at 37 °C for 1 hour to dry. Membranes were then blocked in 1% non-fat dry milk powder, (Omniblok™, American Bioanalytical, Inc., Natick, MA) in PBS buffer, pH 7.2 with shaking for 30 minutes. Membranes were then removed from the blocking solution, blotted dried, and then placed in PBS buffer, pH 7.2, for 5 minutes with shaking to wash. Membranes were removed from the wash solution, blotted dry, and further dried in a vacuum oven at 37 °C for 1 h. The membranes were removed, and an absorbent pad (CF5; Whatman International) was adhered to 1.5 cm PET overhang using 3M 465 adhesive transfer tape. The membrane was then cut into 4 mm test strips.

MBP lateral flow strips were constructed following the method described for the T7 lateral flow strips, except for the use of MBP polyclonal antibody PA1-989 (ThermoFisher Scientific) for the test line. The MBP polyclonal antibodies were at a concentration of 1 mg/mL in PBS without glycerol, and therefore did not require dialysis. 19 µL of the antibody solution was used to achieve a concentration of 0.2 µg of antibody per mm of strip.

ALP lateral flow strips were constructed following the method described for the T7 lateral flow strips, except for the use of a polyclonal antibody ab7321 (Abcam, Cambridge, MA) against *E. coli* alkaline phosphatase for the test line. The ALP polyclonal antibodies were at a concentration of 10 mg/mL and were diluted in PBS to concentration of 1 mg/mL. 19 µL of this antibody dilution was used to achieve a concentration of 0.2 µg of antibody per mm of strip.

5.3.4 Lateral Flow Assay Procedure

The T7 LFA was performed as follows: 50 µL of sample and 5 µL of 5% non-fat dry milk in PBS were placed in a small glass test tube, and the end of a test strip was then placed in the solution. Following absorption of the sample solution, the test strip was transferred to a glass tube containing 30 µL of PBS buffer, pH 7.2, resulting in a wash step. Once the buffer was absorbed, the test strip was placed on a heat block (50 °C) for 20 minutes. The heating step was found to be necessary in order to expose the target peptide sequence of the reporter antibody. A 10 µL solution containing the biotinylated T7 monoclonal capsid antibody (EMD Millipore) diluted 1:200 in PBS, pH 7, was then run up the strip, followed by 30 µL of PBS, pH 7.2, as a wash. Lastly, 5 µL of a 1:200 dilution in PBS of streptavidin conjugated QDot 605® (ThermoFisher Scientfic), was then run up the strip followed by 30 µL of PBS, pH 7.2, as a final wash.

The MBP LFA was performed as follows: 50 µL of sample was incubated with 10 µL of biotinylated polyclonal MBP antibody diluted 1:200 in PBS, pH 7.2 for 10 minutes. The

biotinylated polyclonal MBP antibody was prepared using the EZ-Link™ Sulfo-NHS-Biotin kit (ThermoFisher Scientific) and is the same polyclonal MBP antibody used for the strip test line. After the incubation step, the sample was combined with 5 µL of PBS buffer, pH 7.2, containing 2.5% non-fat dry milk 0.25% Tween 20 (ThermoFisher Scientific) in a small glass test tube, and the end of a test strip was placed in the solution. Following absorption of the sample solution, the test strip was transferred to a glass tube containing 30 µL of PBS buffer, pH 7.2, as a wash. Next, 5 µL of a 1:200 dilution in PBS of streptavidin conjugated QDot 605® (ThermoFisher Scientific), was run up the strip followed by 30 µL of PBS, pH 7.2, as a final wash.

The ALP LFA was performed as follows: 50 µL of sample was added to a small glass test tube, and the end of a test strip was placed in the solution. Following absorption of the sample solution, the test strip was transferred to a glass tube containing 30 µL of PBS buffer, pH 7.2, as a wash. Finally the test strip was transferred to a glass tube containing, 1 mL of 1-Step™ NBT/BCIP Substrate Solution (ThermoFisher Scientific) pre-warmed to 37 °C, and incubated for two hours at 37 °C before reading.

5.3.5 Bacterial Limit of Detection Using $T7_{MBP}$ and $T7_{ALP}$

Three separate overnight cultures of *E. coli* BL21 were serially diluted in LB to achieve 10^3, 10^2, and 10^1 CFU/mL. A 900 µL aliquot of each dilution and a negative control of LB, containing no bacterial cells, were placed in wells of a 96-well DeepWell™ plate, and mixed with 100 µL of 10^3 PFU/mL of $T7_{MBP}$ or 10^4 PFU/mL $T7_{ALP}$ in LB. The samples were incubated at 37 °C with 200 rpm shaking for 5 hours for $T7_{ALP}$ and 7 hours for $T7_{MBP}$ and $T7_{ALP}$. Samples were then passed through a 4mm 0.45 µm Millex®-HV filter, stored at 4 °C, and tested as previously described using either the MBP or ALP lateral flow test trips, with exception that 100 µL of

sample, rather than 50 µL, was run up the strip. The overnight cultures were serially diluted and plated on LB agar to confirm bacterial concentration.

5.3.6 Bacterial Detection in River Water Using $T7_{ALP}$

River water, 2 L, was collected from Fort River in Amherst, MA and autoclaved. Three separate overnight cultures of *E. coli* BL21 were diluted and used to inoculate three separate 300 mL aliquots of water at a target cocentration of 100 CFU/100 mL. 100 mL of water from each aliquot was then passed through a 0.22 µm SCFA filter, and 1 mL of LB was pulled back through the filter to re-suspend the captured cells. Each sample was added to a 15 × 100 mm glass test tube, resulting in three 1 mL tubes corresponding to each of the initial 300 mL river water aliquots. Then 100 µL of 10^5 PFU/mL of $T7_{ALP}$ in LB was added to one tube, and 100 µL of LB to the second, the contents of the third tube were plated to confirm level of *E. coli* recovery. The samples were incubated at 37 °C with 200 rpm shaking for 7 hours. Samples were then passed through a 4mm 0.45 µm Millex®-HV filter, stored at 4 °C, and tested as previously described using the ALP lateral flow test trips with exception that 100 µL of sample, rather than 50 µL, was run up the strip. The overnight cultures were serially diluted and plated on LB agar to confirm bacterial concentration.

5.3.7 Image and Statistical Analysis

Pictures of test strips were then taken using a digital camera, or in the case of fluorescent reporters, a CCD imaging station (Kodak, Rochester, NY, USA). Signal analysis, area under the curve, for the fluorescent reporters was performed using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). The $\log_{10}$ of all test line signals was taken and then evaluated for statistically significance in Origin Pro version 9.0.0 (Northampton, MA). All test line signals

were normalized to the test line signal of the negative control for the run, and then analyzed for significance using a single-sample, one-sided t-test, mean <=1, with an alpha level of 0.05. In all figures error bars represent one standard deviation (SD) ± from the sample mean and a star (*) indicates a significance ($p < 0.05$).

5.4 Results and Discussion

5.4.1 Impact of Phage-Cell Concentration and Time Dynamics

A matrix was used to investigate the impact of starting cell concentrations, starting phage concentration, and incubation time on the dynamics of phage amplification. *E coli* BL21 cells at concentrations of 0, 10^0, 10^1, 10^2, and 10^3 CFU/mL were incubated with 10^1, 10^2, 10^3, and 10^4 PFU/mL of wild-type T7 phage and titered after 3, 5, and 8 hours. The resulting phage titers can be found in Figure 5.1. We observed maximum phage amplification levels of $10^{5.5}$, $10^{8.5}$, and $10^{9.0}$ PFU/mL after 3, 5, and 8 hours of incubation, respectively, when starting with a cell concentration of 1000 CFU/mL. We saw no detectable phage amplification with 3 hours of incubation when starting cell concentration were 100 CFU/mL, though observed a $10^{7.5}$ PFU/mL max after 5 hours, and a $10^{8.8}$ PFU/mL max after 8 hours. Again with 10 CFU/mL, we saw no detectable phage amplification with 3 hours of incubation, but observed a $10^{6.0}$ PFU/mL max after 5 hours, and $10^{8.1}$ PFU/mL after 8 hours. With a 1 CFU/mL starting concentration we observed a $10^{6.2}$ PFU/mL after an 8 hour incubation, and did not detect phage amplification for the other time points. These maximum set the minimum level of sensitivity required for a phage amplification-based LFA, and provides us with some guidance for incubation time. For example, depending on the sensitivity of the LFA, we could potentially detect amplification after an 8 hour incubation for all initial cell levels. Conversely, we could not reasonable expect to detect 100

CFU/mL *E. coli* or less within 3 hours using this scheme as no observable phage amplification occurred during that time. This does not necessarily mean no amplification occurred, only that if it did, the amount of phage did not greatly rise above the level of our initial phage inoculum.

When using phage amplification as part of a detection scheme, it is important to note the impact of initial phage levels on final signal production. By increasing initial phage concentrations the likelihood of infection increases and thus the speed of amplification is similarly increased [153]. This can be seen in Fig. 1 where we detected an increase in phage levels in as little as three hours when initial cells levels were 10^3 CFU/mL. Total phage produced, however, must be sufficient achieve a detectable signal by the assay being used. We found that amplification levels were lower when our initial phage inoculum was at the highest level tested, 10^4 PFU/mL. A similar inverse relationship between starting and final phage levels has also been observed with other phage strains [154]. This is likely due to the dynamics underlying the interactions between cell replication and phage infection. Final phage levels are directly correlated to the total number of cells infected, the more cells there are the more phage produced. Lower initial phage concentrations result in slower infection rates. Additionally, it provides time for uninfected cells to double resulting in an increase in the total amount of cells available for infection and final phage levels, assuming that the time is sufficient to allow all cells to be infected. It is important to note that when using phage amplification to increase the signal from viable cells, a high inoculum titer is not necessarily better.

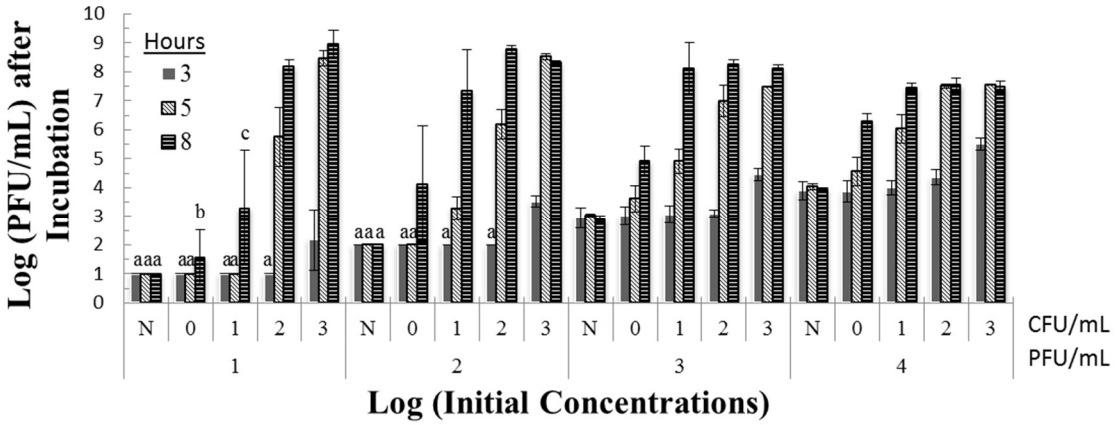

Figure 0.14 Influence of initial phage and bacterial concentrations on phage-amplification with respect to time. [a]Titer of all replicates were Too Few To Count, indicating no amplification had occurred during incubation. [b]Titer of two replicates was TFTC, indicating no amplification occurred in those samples. [c]Titer of one replicate was TFTC, indicating no amplification occurred in one sample. In the cases of TFTC, the initial PFU/ml was used for calculate average and standard deviation. Error bars represent one SD ± from the sample mean.

5.4.2 Lateral Flow Assay Limit of Detection

We compared the limit of detection of three phage-amplification based lateral flow assays (Figure 5.2). The first assay targeted the T7 phage virion itself, and used a fluorescent quantum dot reporter. The second assay targeted MBP, a peptide co-produced during $T7_{MBP}$ amplification, also using a fluorescent quantum dot reporter. The third assay targeted alkaline phosphatase, an enzyme that is overproduced during $T7_{ALP}$ amplification, and whose activity was detected using the substrate BCIP/NBT, which results in the production of a visible, dark blue precipitate.

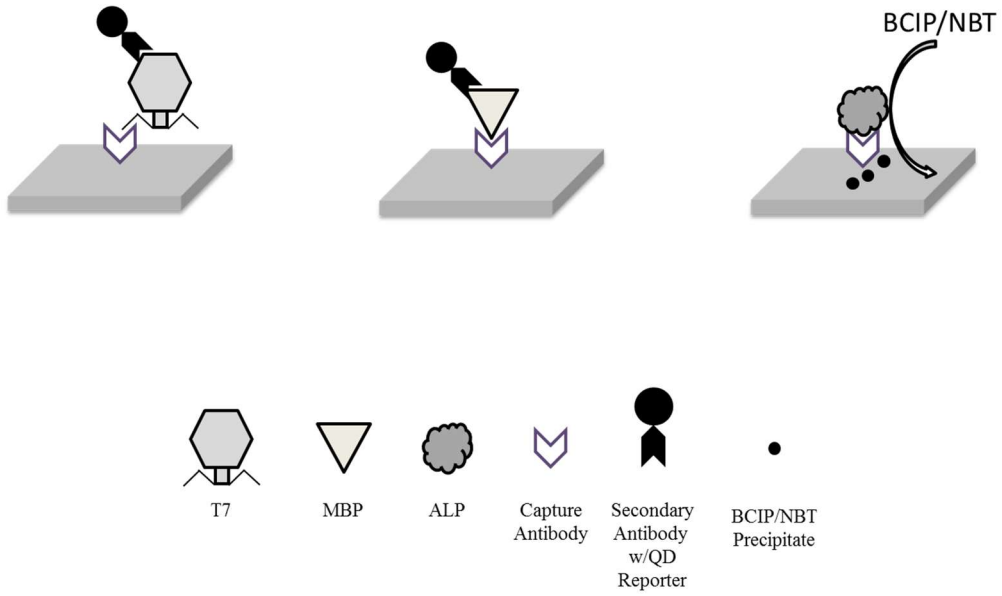

Figure 0.15 Lateral flow assay (LFA) Schematic. The LFA consists of a nitrocellulose membrane with a test line containing capture antibodies against either: (i) the tail fiber of T7; (ii) maltose binding protein; or (iii*) E. coli* alkaline phosphatase. Biotinylated secondary antibodies against either: (i) the leader sequence of capsid protein 10B of T7; or (ii) maltose binding protein; in combination with a streptavidin conjugated quantum dot reporter are used to produce a signal at the test line. In the case of alkaline phosphatase strip, the membrane is placed in BCIP/NBT, and if phosphatase is present a dark blue precipitate is deposited at the test line.

Dilution series of stock T7, $T7_{MBP}$, and $T7_{ALP}$ were prepared in triplicate and tested using

the corresponding lateral flow assay. The reporter signal produced at the test line was proportional to the corresponding phage titer of the sample and therefore used to determine the minimum level of phage amplification necessary to produce a signal. The test line signal of each dilution series sample were analyzed using ImageJ and compared to the signal at the test line of the negative control strip for the series (Figure 5.3a). A representative set of strips can be found in Figure 5.3b, these images were adjusted for brightness and contrast in order to better visualize the test line. For T7 assay, while one can visually distinguish a test line in samples containing at least $10^{6.8}$ PFU (Figure 5.3b), statistically significant fluorescent signals were only observed at the test line in samples containing at least $10^{7.8}$ PFU or wild type T7 (Figure 5.3a). With the MBP lateral flow assay, we were able to visually distinguish and detect a statistically significant test line signal in samples containing at least $10^{6.2}$ PFU of $T7_{MBP}$ (Figure 5.3). At the highest concentration tested, $10^{8.2}$ PFU, a statistically significantly signal was not detected ($p = 0.0502$). Lastly with the ALP lateral flow assay we were able to visually distinguish and detect a

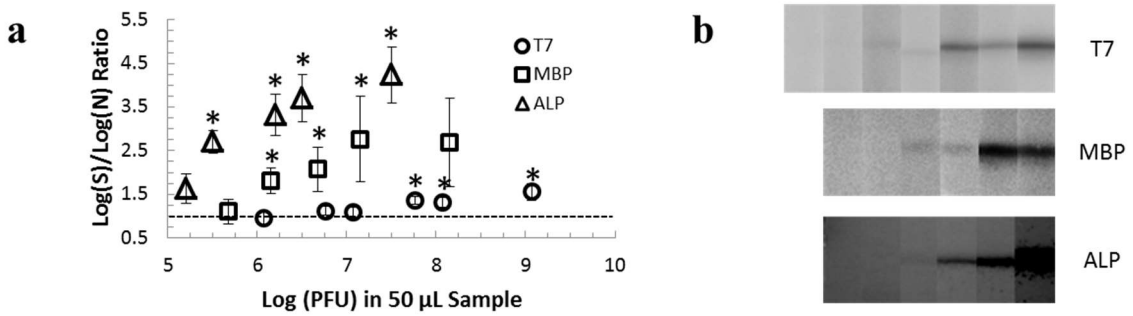

Figure 0.16 LFA Limit of Detection. (a) 50 µL samples of a dilution series of wild-type T7, $T7_{MBP}$, and $T7_{ALP}$ stock were tested using the T7 LFA, MBP, and ALP LFA, respectively. Sample test line signals were normalized to the negative control test line signal (S/N) for all assays using the quantum dot reporter. The dotted line denotes an S/N ratio = 1. Error bars represent one standard SD ± from the sample mean and a star (*) indicates a significance ($p < 0.05$). (b) Representative strips of T7, $T7_{MPB}$, and $T7_{ALP}$ LFA trials. The left most strip corresponds to the negative control, and each subsequent strip is increasing phage titer corresponding to the respective data points in part a.

statistically significant test line signal in samples containing at least $10^{5.5}$ PFU of $T7_{ALP}$ (Figure 5.3). Based on a sample volume of 50 µL, the final phage amplification levels required for detection were calculated to be: $10^{9.1}$ PFU/mL of wild-type T7; $10^{7.5}$ PFU/mL of $T7_{MBP}$, and $10^{6.8}$ PFU/mL of $T7_{ALP}$. Thus using a peptide reporter produced by a bioengineered T7, we lowered the required phage-amplification levels for a detectable signal by 1.6 $\log_{10}$, a great than 10-fold increase in sensitivity. Using the alkaline phosphatase reporter further reduced necessary phage-amplification levels by a total of 2.3 $\log_{10}$, greater than 100-fold improvement in sensitivity. These results highlight the potential of targeted bioengineering of phage to produced reporter molecules that can enhance the performance of phage amplification-based LFAs.

5.4.3 Detection of *E. coli* in Broth Using $T7_{MBP}$ and $T7_{ALP}$ Amplification-based Lateral Flow Strips

As previously noted, the phage amplification matrix (Figure 5.1) provided guidance on the maximum phage amplification we could expect over time based on starting cell concentrations. While we were able to detect phage virions using the T7 LFA, none of phage amplification observed surpassed the $10^{9.1}$ PFU/mL limit of detection for the T7 LFA. This suggests, at when cells concentrations are 1000 CFU/mL, that a T7-targeted LFA would not be adequate for successful detection of *E. coli*.

The phage amplification matrix also indicated that a starting cell concentration of 1 CFU/mL would not result in phage amplification sufficient to trigger detection in either of our bioengineered phage-based LFAs. For the MBP reporter, which has a detection limit of $10^{7.5}$ PFU/mL, there was one case where a starting cell level of 10 CFU/mL resulted in an average phage amplification level greater than $10^{7.5}$ PFU/mL, however there was a lot variability for that data point. We saw maximum phage amplification levels after 8 hours for starting cell

concentrations of 100 and 1000 CFU/mL with an initial phage inoculum level of 10^2 PFU/mL, so we chose this inoculum level for further $T7_{MBP}$ testing. We saw phage amplification greater than $T7_{ALP}$ LFA limit of $10^{6.8}$ PFU/mL for initial cell concentrations of 10 CFU/mL and greater, when initial phage inoculum levels were 10^4 PFU/mL. We therefore chose this inoculum level for further $T7_{ALP}$ testing.

We incubated several concentrations of *E. coli* BL21, from 10^1 to 10^3 CFU/mL, and a negative control of LB in the absence of target bacteria, at 37 °C with an inoculum of either $T7_{MBP}$ or $T7_{ALP,}$ for 7 hours for the former, and 5 and 7 hours for the later. At the end of incubation, we filtered the samples to remove cellular debris, and ran a 100 µL aliquot up the appropriate lateral flow strip as previously described. Note, an added benefit of doubling the volume of the sample aliquot to 100 µL, is that it lowers the amount of phage amplification needed to be reached in the sample by 0.3 $\log_{10}$, i.e for $T7_{ALP}$, the necessary level went from $10^{6.8}$ PFU/mL to $10^{6.5}$ PFU/mL and from $10^{7.5}$ to $10^{7.2}$ for $T7_{MBP}$. A statistically significant signal from phage amplification could be detected at the test line from samples with an initial bacterial concentration of 10^3 CFU/mL for both $T7_{MBP}$ after 7 hours and $T7_{ALP}$ after 5 hours (Figure 5.4a). We were able to detect a statistically significant signal from $T7_{ALP}$ amplification after a 7 hour incubation from a starting concentration of 10^2 and 10^3 CFU/mL of *E. coli* (Figure 5.4a). Based on the phage amplification matrix (Figure 5.1), we had expected to see signal from $T7_{ALP}$ amplification at 10^1 CFU/mL, but did not. It is possible that both the 1 hour decrease in incubation was sufficient to prevent amplification levels from reaching those observed within 8 hours in our earlier matrix. It is also possible that the insertion of alkaline phosphatase into the T7 genome, and its subsequent overexpression during infection could lower the amplification efficiency of the bioengineered $T7_{ALP}$ in comparison to the wild type used for the matrix, and

thus not achieve the same level of amplification. The $T7_{ALP}$ strips were allowed to develop in BCIP/NBT overnight, and a visually detectable line did form at the test line for 10^1 CFU/mL samples (data not show).

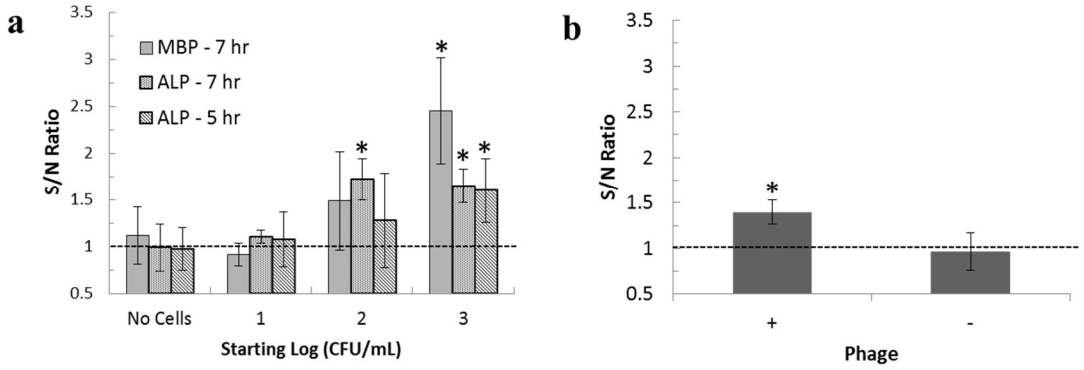

Figure 0.17 Bacterial Detection. (a) Samples of each *E. coli* concentration were tested with the corresponding LFA following either a 5 or 7 hour incubation at 37 °C with $T7_{ALP}$ or $T7_{MBP}$; (b) River water containing 100 CFU/100 mL of *E. coli* was filter concentrated and cells re-suspended in LB with (+) or without (-) $T7_{ALP}$ and incubated for 7 hours at 37 °C. 100 µL of each samples were tested with the ALP LFA. In both graphs sample test line signals were normalized to the negative control test line signal (S/N). The dotted line denotes an S/N ratio = 1. Error bars represent one SD ± from the sample mean and a star (*) indicates a significance ($p < 0.05$).

5.4.4 Proof-of-Principle Detection of *E. coli* in river Water Using $T7_{ALP}$ Amplification-based Lateral Flow Strips

E. coli is commonly used as an indicator of drinking and recreational water quality[155], and the rules for produce safety proposed by the FDA as part of the Food Safety Modernization Act (FSMA) will also establish microbiological quality criteria for agricultural water. These produce safety rules will require regular water monitoring for generic *E. coli* with a rolling 5-sample mean limit of 126 CFU/100 ml and a statistical threshold value of 410 CFU/100 mL[156]. While there are many commercially available tests to detect *E. coli* in water, the majority of these tests take anywhere from 24-48 hours to provide results[36]. This time delay presents significant challenges

for farmers in regards to water use management and appropriate corrective action response to the discovery of non-complaint water.

Our bacterial limit of detection using $T7_{ALP}$ amplification was 100 CFU/mL, essentially two orders of magnitude higher that the FSMA agricultural water limit. Filtration of 100 mL is a commonly used method for bacterial concentration when performing *E. coli* testing of water. To explore whether this concentration step would help us to reach the essentially 1 CFU/mL FSMA limit, we took 100 mL samples of sterilized river water and spiked them with ~100 CFU of *E. coli* strain BL21, passed them through a 0.45 µm filter to capture the cells. We pulled 1 mL of LB back through the filter to re-suspend the cells and inoculated with $T7_{ALP}$. To further demonstrate that any positive signal was due to alkaline phosphatase production from $T7_{ALP}$ amplification and not due to alkaline phosphatase endemic to *E. coli,* our negative controls were samples spiked with *E. coli* that were not subsequently inoculated with phage. Samples were incubated for 7 hours at 37 °C. A 100 µL aliquot of each of these samples was tested using the $T7_{ALP}$ LFA, and the strips developed in BCIP/NBT for 2 hours. Pictures were taken and analyzed for signal. We were able to detect a statistically significant signal at the test line in the samples inoculated with $T7_{ALP}$, and detected no signal in negative controls (Figure 5.4b). This proof-of-principal demonstrates the potential for phage amplification-based LFAs to rapidly detect regulatory relevant levels of bacteria*,* though further work is needed to explore the impact of other microbial organisms that would be co-concentrated in the sample.

5.5 Conclusion

Our goal was to investigate the use of bioengineered phage to improve the sensitivity of phage-amplification based lateral flow assays for the detection of bacteria. We demonstrated that by bioengineering phage to overexpress peptide and enzymatic reporters, that we could

improve the sensitivity of phage-amplification based LFA by over 100-fold. This increase in sensitivity enabled the detection 10^3 CFU/mL of *E. coli* after 7 hours, and a regulatory limit of 100 CFU/100 mL of *E. coli* when combined with a filter concentration step in 9 hours. The LFA format is ideal for use in low-resource settings, such as some clinics, farms, and food production facilities; and modern molecular techniques allow for the replication of our alkaline phosphatase reporter scheme in other phage to ensure broad species coverage and allows for the targeting of other bacterial species of significance. Further research into LFA-amenable phage-delivered reporter peptides and enzymes will unlock the power and sensitivity of phage-based diagnostics, allowing for the development of novel and versatile bacterial detection assays.

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